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STRESS GRANULES MODULATE SYK TO CAUSE MICROGLIAL DYSFUNCTION IN ALZHEIMER'S DISEASE

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STRESS GRANULES MODULATE SYK TO CAUSE MICROGLIAL DYSFUNCTION IN ALZHEIMER'S DISEASE

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To my parents and my best friend,

Anukana

who never stopped in believing and supporting me.

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AMPK	5' adenosine monophosphate-activated protein kinase
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
AP-1	Activator protein-1
ATP	Adenosine triphosphate
AD	Alzheimer's disease
APP	Amyloid precursor protein
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
ATG	Autophagy related protein
AUB	AU-binding proteins
BCR	B-cell receptor
Aβ	Beta-amyloid
β-CTF	Beta C-terminal fragments of APP
BDNF	Brain derived neurotrophic factor
BAI1	Brain-specific angiogenesis inhibitor 1
BRF1	B-Related Factor 1
BTK	Bruton's tyrosine kinase
CAMKK2	Calcium /calmodulin dependent kinase kinase 2
CaMKII	Calcium/ Calmodulin-dependent protein kinase II
CREB	cAMP response element binding protein
CNS	Central nervous system
CX3CL1	Chemokine fractalkine
JNK3	c-Jun N-terminal kinase 3
CSF1R	Colony stimulating factor 1 receptor
Cdk5	Cyclin dependent kinase 5
COX2	Cyclooxygenase 2
CNTF	Cytokine ciliary neurotrophic factor
CPEB	Cytoplasmic polyadenylation element binding protein
DAMPs	Damage associated molecular patterns
DAP12	DNAX-Activation Protein 12
Elk-1	E26-like kinase 1
EAD	Early-onset AD
EPHB2	Ephrin receptor B2
EMPs	Erythromyeloid progenitors
eIF2α	Eukaryotic translation initiation factor 2-alpha
ERK	Extracellular-signal-regulated kinases

FASL	Fas Ligand
FcεR1	Fc Epsilon receptor 1
FBP	Folate-binding protein
FMRP	Fragile X mental retardation protein
FXR1	Fragile X mental retardation related protein 1
FLD	Frontotemporal lobar degeneration
FTLD	Frontotemporal lobar degeneration
FUS	Fused in Sarcoma
GCN2	General control nonderepressible 2
GWAS	Genome wide association studies
GPX	Glutathione peroxidase
GST	Glutathione S-transferase
GSH	Glutathione
GW182	Glycine-tryptophan 182
GSK-3β	Glycogen synthase kinase 3 beta
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRB7	Growth factor receptor-bound protein 7
HRI	Heme-regulated eIF2α kinase
HDLS	Hereditary Diffuse Leukoencephalopathy with Spheroids
HDAC6	Histone deacetylase 6
HD	Huntington's disease
H_2O_2	Hydrogen peroxide
OH^-	Hydroxyl anion
IgG	Immunoglobulin G
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
iNOS `	Inducible nitric oxide synthase
IP5K	Ins(1,3,4,5,6)P5 2-kinase
IGF-1	Insulin-like growth factor 1
IFNɣ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-1	Interleukin-1
LAD/ LOAD	Late-onset AD
LAT	Linker for activation of T cells
LPS	Lipopolysaccharide
MCP	Macrophage chemotactic protein
M-CSF	Macrophage-colony stimulating factor
mTOR	Mammalian target of rapamycin
MTK1	MAP kinase kinase kinase
mRNA	Messenger RNA
MG	Microglial cell
MCI	Mild cognitive impairment
MFG-E8	Milk fat globule epidermal growth factor 8
mmLDL	Minimally oxidized low-density lipoprotein
MAPK	Mitogen activated protein kinase

MEGF10	Multiple epidermal growth factor-like domains 10
MS	Multiple sclerosis
NGF	Nerve growth factor
NPC	Neuronal precursor cells
NT-3	Neurotrophin 3
NO	Nitric oxide
NMDA/ NR2B	N-methyl D-aspartate receptor
NSAID	Nonsteroidal anti-inflammatory drugs
NFAT	Nuclear factor of activated T cells
PNS	Peripheral nervous system
$ONOO^-$	Peroxynitrite
PS	Phosphatidylserine
PI3K	Phosphoinositide 3-kinase
PDK1	Phosphoinositide-dependent kinase-1
PLCɣ	Phospholipase Cɣ
pTYR	Phosphotyrosine
PABP1	Poly(A)-binding protein 1
PSEN1/ PSEN2	Presenilin 1/ 2
PRNP	Prion protein
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PKR	Protein kinase R
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PP-2A	Protein phosphatase 2A
G3BP	Ras-GTPase-activating protein SH3-domain-binding protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RACK1	Receptor for activated C kinase 1
RAGE	Receptor for advanced glycation endproducts
RNP	Ribonucleoprotein
RAP55	RNA-associated protein 55
SLP76	SH2 domain leukocyte-specific phosphoprotein
SIGLECS	Sialic acid-binding immunoglobulin superfamily lectins
SIRPβ1	Signal regulatory protein-β1
SA	Sodium arsenite
sAβ	Soluble Aβ
SYK	Spleen tyrosine kinase
SH2	Src Homology 2
STAU1	Staufen1
SRC-3	Steroid receptor coactivator-3
SG/ SGs	Stress granules
O_2^-	Superoxide
SMN	Survival of motor neuron
TIA-1	T cell internal antigen-1
TIAR	T cell internal antigen-1-related

TDP-43	TAR DNA-binding protein 43
TIM4	T-cell immunoglobulin mucin receptor 4
TRAF2	TNF Receptor-Associated Factor 2
TLRs	Toll like receptors
TREM2	Triggering Receptor Expressed On Myeloid Cells 2
UPS	Ubiquitin-dependent proteasome system
TTP	Tristetraprolin
TNF-α	Tumor necrosis factor α
TYROP	TYRO Protein Tyrosine Kinase Binding Protein
VCP	Valosin-containing protein
ZBP1	Z-DNA Binding Protein 1
ZAP70	ζ-chain-associated protein kinase

ABSTRACT

Ghosh, Soumitra. PhD., Purdue University, December 2015. Stress Granules modulate Syk to cause Microglial Dysfunction in Alzheimer's disease. Major Professor: Robert L. Geahlen.

Microglial cells in the brains of Alzheimer's patients are recruited to amyloid beta (Aβ) plaques and exhibit an activated phenotype, but are defective for plaque removal by phagocytosis. To explore the molecular basis for these phenomena, I hypothesized that defects in the functions of the protein-tyrosine kinase SYK, which is important both for macrophage activation and phagocytosis, might underlie much of this pathology. Recent evidence from our lab indicates that SYK can associate with stress granules, ribonucleoprotein particles that form in stressed cells and contain inactive translation initiation complexes. I found that microglial cell lines and primary mouse brain microglia, when stressed by exposure to sodium arsenite or Aβ(1-42) peptides or fibrils, form extensive stress granules to which the tyrosine kinase, SYK, is recruited. SYK enhances the formation of stress granules as evidenced by the inhibition of stress granule formation by small molecule inhibitors, knockdown of SYK expression by shRNA and *SYK* haploinsufficiency in mouse microglial cells. SYK is active within the resulting stress granules where it catalyzes the phosphorylation of stress granule-associated proteins on tyrosine. SYK-dependent stress granule

formation stimulates the production of reactive oxygen and nitrogen species. These are toxic to neuronal cells as demonstrated by a co-culture assay using stressed microglial cells and HT22 neuronal cells. The ability of microglial cells to phagocytose *E. coli* is blocked by SYK inhibitors. The sequestration of SYK into stress granules inhibits the ability of microglial cells to phagocytose either *E. coli* or Aβ fibrils. Microglial cells from aged mice are more susceptible to the formation of stress granules than are cells from young animals. Stress granules containing SYK and phosphotyrosine are prevalent in the brains of patients with severe Alzheimer's disease, suggesting that the sequestration of SYK into stress granules is part of the pathology of the disease. Phagocytic activity can be restored to stressed microglial cells by treatment with IgG independent of the epitope specificity, suggesting a mechanism to explain the therapeutic efficacy of intravenous IgG.

CHAPTER ONE: INTRODUCTION

1.1 **Introduction**

Microglial cells (MG) are the professional macrophages of the central nervous system. Among their many tasks is the removal from the brain of aggregates of β-amyloid (Aβ), which is formed from a proteolytic product of amyloid precursor protein (1). The persistent accumulation of β-amyloid plaques is a characteristic of Alzheimer's disease (AD), a serious neurodegenerative disorder affecting millions of patients worldwide. MG are attracted to sites of Aβ deposition, which they recognize through a variety of cell surface receptors, and are capable of Aβ removal through phagocytosis (2, 3). Impaired microglial activity, as exhibited by defective removal of Aβ plaques, is particularly associated with the later stages of AD as plaques accumulate (4-6). These plaques still attract MG, but are refractory to phagocytosis. While MG can phagocytose Aβ fibrils, they also become activated as a consequence of receptor engagement by Aβ (7-9). The activation of MG is problematic as the resulting inflammatory response can damage neighboring neuronal cells (9). Compelling evidence for an important role of MG in AD comes, in part; from large scale studies of genes associated with AD that directly implicate inflammatory responses of MG as critical for AD pathology. Products of genes with identified associations with AD include Triggering Receptor Expressed on Myeloid

Cells 2 (TREM2), TYRO Protein Tyrosine Kinase Binding Protein (TYROBP) and CD33 (10-15). The myeloid receptor TREM2 functionally associates with TYROBP and promotes the internalization of bacteria and apoptotic neurons and recruits MG to Aβ plaques (16-19). Loss-of-function mutants promote inflammatory responses, decrease phagocytosis and predispose patients to AD (10). CD33 is dysregulated in MG in AD brain and is associated with reduced uptake of Aβ and increased numbers of activated, pro-inflammatory MG (20).

Many phagocytic receptors contain or are associated with proteins such as TYROBP that contain immunoreceptor tyrosine-based activation motifs (ITAMs) (21, 22). Receptor engagement initiates the phosphorylation of the two ITAM tyrosines leading to a physical interaction with the tyrosine kinase, SYK (23). Signaling through ITAM receptors is attenuated by receptors with immunoreceptor tyrosine-based inhibitory motifs (ITIMs) such as CD33 (24). Since SYK is essential for both phagocytosis in macrophages and for the receptor-mediated activation of inflammatory responses (25, 26), two events that are disrupted in MG from AD brains, it would appear to be a prime candidate for a critical mediator of AD pathology. An important question is how the function of SYK might be disrupted in aged or stressed MG.

MG in aged and AD brain fail to clear Aβ plaques, but display an activated, pro-inflammatory phenotype (4-6). How aging and other stresses affect MG to alter their homeostatic functions is unclear. Eukaryotic cells respond to external stresses via the formation of ribonucleoprotein (RNP) complexes scaffolded by RNA-binding proteins that self-associate. Examples of RNP aggregates include stress granules (SGs), which contain stalled initiation complexes and their associated mRNAs (27, 28). An association

of RNP aggregates with neurodegenerative diseases is well documented and mutations in RNA-binding proteins that promote self-assembly can be drivers of motor neuron diseases (29, 30). Similarly, defective clearance of SGs leads to the pathological accumulation of RNP particles and underlies the pathology of amyotrophic lateral sclerosis, Huntington's disease (HD), frontotemporal lobar degeneration (FLD) and AD (31-40).

Large scale proteomic screens from our laboratory have identified multiple SG-associated proteins as binding partners and substrates for SYK (41-43). SYK is recruited to SGs in some cells when exposed to sodium arsenite (SA) or proteasome inhibitors. In this study, I find that SYK also is recruited to SGs that form in MG, not only in response to sodium arsenite, but also in response to Aβ peptides or fibrils. Chronic stress leads to the formation of persistent SGs in MG in which active SYK is sequestered, leading to the constitutive production of reactive metabolites and the inhibition of phagocytic activity. Cytoplasmic aggregates containing SYK and phosphotyrosine are more pronounced in MG in the brains of patients with advanced AD. Based on this information, I propose a model in which the production of SGs and the corresponding misregulation of SYK directly contributes to AD pathology. Interestingly, the impaired phagocytic activity of stressed MG can be restored by the induced relocation of SYK from the SG to the plasma membrane by treating cells with Immunoglobulin G, suggesting a mechanism by which the administration of immunoglobulins to AD patients might retard disease progression.

1.2 **Alzheimer's disease and its causes**

Alzheimer's disease (AD) is one of the most prevalent neurodegerative disorders in the world. According to recent statistics, five million Americans are living with AD in 2015, a number predicted to rise to sixteen million by 2050 (44). A substantial increase in the older population is also considered a major contributor to this alarming increase in AD patients. AD is categorized into two categories: early-onset AD (EAD) and late-stage AD (LAD). Mild-cognitive impairment (MCI) is a cognitive dysfunctional condition that precedes AD and contributes to its progression (45-48). Currently we do not have a method to detect AD in its early stages (49). The clinical symptom of AD develops much later than the initial onset of the disease; hence early detection is extremely difficult. The primary cause of dementia in AD is still unknown (50). The term AD was first coined in 1910 by Dr. Kraepelin following its discovery in 1906 by Dr. Alois Alzheimer. Considerable progress in understanding the complexity of the disease has not yet led to a successful therapeutic intervention. Hence we do not have a successful drug candidate to cure AD.

1.3 **Amyloid beta and Tau pathology**

The patients suffering from AD encounter reduced memory, cognition dysfunction and massive neuronal cell death over time. Post mortem examination of AD patients' brains have revealed amyloid beta (Aβ) plaques and tau fibrillary tangles as the two hallmarks of AD (10, 45, 46, 51-53). Aβ aggregates into oligomers, fibers and plaques in the

diseased brain that hinder the normal functioning of brain in various ways (54-59). Aβ is amino acid peptide sequence of variable size (38, 40 and 42 amino acids) that is formed as result of cleavage of amyloid precursor protein (APP) by two enzymes: β-secretase and ɣ-secretase. It is considered that Aβ deposition is an initiating pathological event in AD. Over time Aβ undergoes different stages of aggregation, forming soluble oligomers, insoluble oligomers and insoluble fibrils (60-63). These Aβ deposits impair synaptic functioning resulting in chronic neurodegeneration ultimately leading to excessive neuronal cell death, cognitive impairment and severe dementia (64-67). Targeting Aβ deposition through drugs, antibodies and small molecule inhibitors have proven to be effective in alleviating the symptoms to a certain extent, but not completely (68-73).

Tau is another key protein involved in neurodegeneration. Tau is a microtubule-associated protein found in mature neurons where it stabilizes microtubules. In normal brain, tau is both O-GlcNAcylated and phosphoryled at low levels. In AD brain, hyperphosphorylation of tau leads to impairment of its normal biological activity. In neurodegenerative conditions, tau becomes N-glycosylated; and this glycosylation makes it more prone to phosphorylation by protein kinase A (PKA), glycogen synthase kinase 3 beta (GSK-3β) and cyclin dependent kinase 5 (Cdk5). Additionally protein phosphatase A (PP-2A) activity is decreased, but calmodulin-dependent protein kinase II (CaMKII), PKA and mitogen activated protein kinase (MAPK) activities are increased in the CNS (69, 74-84) . The hyperphosphorylation of tau leads to a breakdown of the microtubule network and subsequent formation of paired helical filaments which make up neurofibrillary tangles. Finally axonal transport is compromised resulting in retrograde degeneration and dementia (77-80, 85).

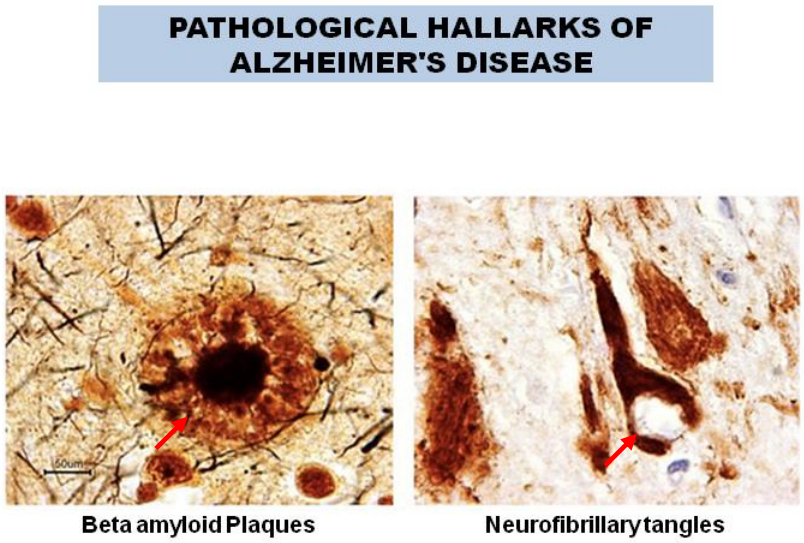

Figure1.1 Schematic diagram of Alzheimer's disease hallmarks

In human AD brain, Amyloid beta protein aggregates to form amyloid plaques as shown in the figure. TAU protein also hyper-phosphorylates to form neurofibrillary tangles as shown above. Adapted from NIA-NIH webpage (Imaged by Colorado Hospital). Scale=5 µm

1.4 **Oxidative stress in AD**

Apart from Aβ and tau pathology, there are several other factors that contribute to AD pathology. Maintenance of cellular redox levels is crucial for the normal functioning of neurons. Studies on AD brains suggest excessive oxidative stress damages neurons and other supporting cells in the brain. Common oxidants such as hydrogen peroxide (H_2O_2), superoxide (O_2-) and peroxynitrite ($ONOO-$) are highly elevated in AD brains (74, 86-89). Additionally there is a decrease in antioxidants such as glutathione (GSH) and its supporting enzymes, glutathione peroxidase (GPX), and glutathione S-transferase (GST) (90). The increase in oxidative stress is mainly attributed to elevated oxidative phosphorylation in damaged mitochondria. AD progression impairs the normal efficiency of the mitochondria and leads to a feed-forward mechanism of superoxide production (91-93). Increase in ROS/ RNS promotes neuroinflammation through increase in cytokines, activation of apoptotic pathways and overexpression of transcription factors leading to neuronal cell death (85, 92, 94-100).

1.5 **Genes, proteins and kinases involved in AD**

AD is also classified into two categories, sporadic and familial, based on its causative origin. Our knowledge about the genes involved in AD mainly comes from studies involving familial AD cases. Studies for decades have revealed that several genes and proteins through elevated levels, increased activity and mutations contribute to AD progression.

Amyloid precursor protein (APP) is a type1 transmembrane protein that is highly expressed in neurons and astrocytes. Proteolytic processing of APP leads to amyloidogenic pathways (101, 102). APP is cleaved to generate Aβ peptides, soluble APPβ and beta C-terminal fragments β-CTF (103, 104). APP on the cell surface is generally internalized allowing Aβ to be generated within the endocytic pathway and then released into the extracellular space (105). Recessive genetic mutations in APP such as A673V and E693Δ are reported to cause early-onset AD (106). Mutations such as the Swedish mutation (KM670/671NL) increases plasma Aβ levels by altering β-secretase enzyme efficiency whereas the Dutch mutation (E693Q) occurs in the Aβ domain and results in excess Aβ aggregation (107, 108).

Presenilin 1 (PSEN1) and presinilin 2 (PSEN2) are integral membrane proteins that are important components of the ɣ-secretase complex, which is responsible for cleaving APP into Aβ fragments (109, 110). Their presence in the endoplasmic reticulum makes them an important component of protein processing. To date, 185 dominant mutations have been identified in *PSEN1* that account for 80% of early-onset familial AD and 13 dominant pathogenic mutations have been identified in *PSEN2* (111, 112).

A Disintegrin and metalloproteinase domain-containing protein 10 **(**ADAM10) is a metalloproteinase domain-containing protein that is considered a major α-secretase and is responsible for cleaving the APP ectodomain. ADAM10 mutants Q170H and R181G increase Aβ levels in vitro and in AD transgenic mice (113-116).

Apolipoprotein E (APOE) is a prime risk gene in AD. It is a pleiotropic glycoprotein that is highly expressed in liver, brain and macrophages. It regulates the mobilization and redistribution of cholesterol. APOE in the brain is required for neuronal

growth, nerve repair and regeneration, immune response and activation of lipolytic enzymes. APOE exists in three different isoforms: APOEε2, APOEε3 and APOEε4. APOEε4 has been reported to increase the risk of familial and sporadic early- and late-onset AD. The increase in APOE expression can increase plaque formation and accumulation (51, 106, 117-119).

Evidence suggests that cellular prion protein PRNP plays an important role in several neurodegenerative diseases including AD. It has a very high binding affinity for Aβ oligomers as confirmed in human AD brains. It binds to the tyrosine kinase FYN, which phosphorylates N-methyl D-aspartate receptor subtype 2B (NR2B) subunit and TAU on tyrosine. Interestingly, FYN phosphorylates TAU postsynaptically to induce Aβ toxicity mediated through the N-methyl-D-aspartate (NMDA) receptor (120-127).

Ephrin receptor B2 (EPHB2) is a tyrosine kinase that has a high affinity for Aβ oligomers and helps in regulating the synaptic localization and function of NMDA receptors. Studies have revealed that Aβ oligomers can bind EPHB2 and lead to its proteosomal degradation. This affects NMDA receptor signaling, resulting in defective long term potentiation (LTP) (66, 120, 128-130).

Evidence has suggested an impairment of calcium signaling in AD brains. Aβ induced calcium elevation activates the calcium-dependent phosphatase calcineurin, which in turn leads to the nuclear localization and increased transcriptional activity of nuclear factor of activated T cells (NFAT). GSK3 phosphorylates NFAT to keep it out of the nucleus which in turn increases Fas ligand (FASL) levels. Calcium /calmodulin dependent kinase kinase 2 (CAMKK2) is a serine /threonine kinase whose activity is directly dependent on calcium influx in the brain (131-138). The CAMKK2- 5' adenosine

monophosphate-activated protein kinase (AMPK) pathway mediates early synaptotoxic effects of Aβ oligomers through Tau phosphorylation (139, 140).

Mitochondrial damage is an early event observed in AD cell–culture and animal models. Centaurin-α1 is an Arf GTPase-activating protein that promotes ADP ribosylation and is required for normal dendritic development. It interacts with RAS and Ras-E26-like kinase 1 (Elk-1) in AD brains. Mitochondrial permeability is directly regulated by centaurin-α1 and Elk-1. Ras-Elk-1 pathway mediated mitochondrial damage leads to loss of dendritic spines and spine structural plasticity (115, 141-148).
5' adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways are directly modulated by Ras and MAPK in neurons leading to neuronal cell death. mTOR is a serine/threonine kinase that plays a critical role in several cellular processes such as maintaining cell size, cell proliferation and autophagy through regulation of protein synthesis. Specifically in neurons, mTOR plays a role in synaptic plasticity, axon pathfinding and regeneration, dendrite arborization and spine morphology maintenance. In AD brains, mTOR signaling is unregulated in both mouse models and human samples. This manifests in an early cognitive decline and reduced autophagy that can be rescued by inhibition of mTOR by rapamycin. Additionally, AMPK and MAPK4 further phosphorylate TAU on S262 in the microtubule-binding domain. This in turn promotes tau aggregation and hinders its clearance (149, 150).

Two other kinases that play an important role are Cdk5 and c-Jun N-terminal kinase 3 (JNK3). ER stress is a common phenomenon observed in AD. ER stress activates JNK3 that further phosphorylates APP. Thus increased JNK3 activity in AD

brain facilitates endocytosis of APP and further processing of Aβ (142, 151-160). Cyclin dependent Kinase (Cdk5) is a proline-directed serine/ threonine kinase that also plays a critical role in AD progression. In neurons, Cdk5 binds to its activator p35 or p39 to maintain its activation. Later in AD, Cdk5 is over activated by the p25 component of p35 and this leads to synaptic failure, which promotes neuronal cell death and loss of learning and memory. This overactivation leads to Tau aggregation, Aβ accumulation, activation of downstream transcription factors, and increased oxidative stress in the brain (90, 152, 161-166).

GSK3β is modulated by Wnt signaling and insulin levels in the brain. GSK3 also reduces acetylcholine synthesis and promotes neuronal apoptosis. GSK3β plays a pivotal role in AD signaling cascade leading to hyperphosphorylation of tau and increased Aβ production along with cerebral inflammation (162, 167, 168).

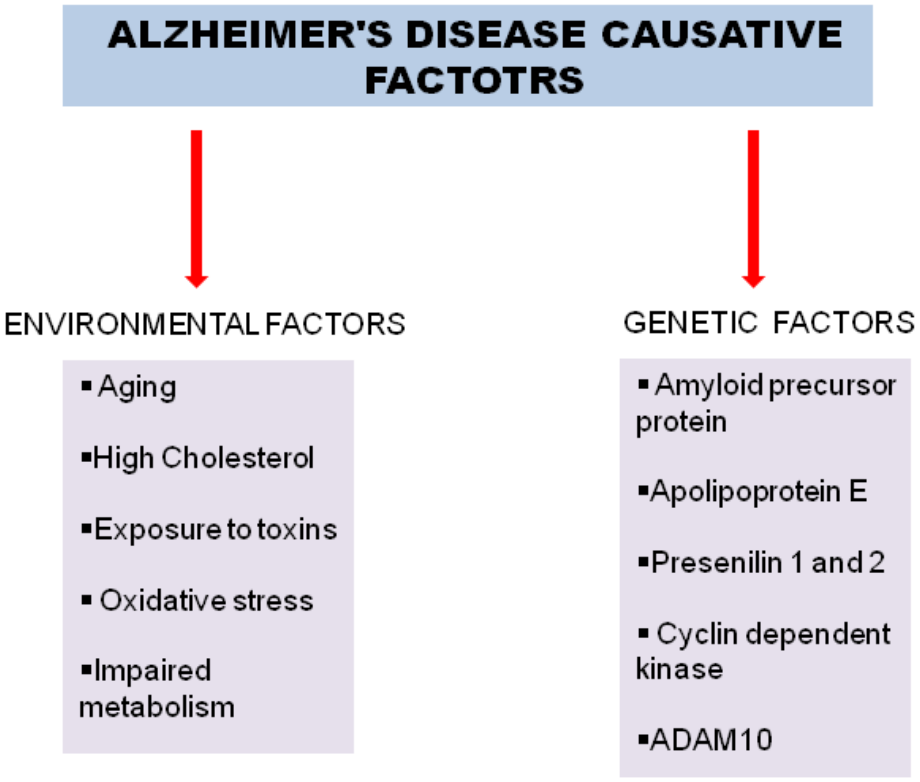

Figure 1.2 Schematic chart of Alzheimer's disease causative factors

In AD, both environmental and genetic factors contribute towards the manifestation of the disease. Environmental factors as mentioned above are considered the cause behind 80% AD cases. These factors could also lead to mutations in genes described in genetic factors. Genetic factors like Amyloid precursor protein interact with other genes and proteins that predispose individual to AD.

1.6 **Neuroinflammation in AD**

Neuroinflammation is a crucial event that contributes to AD pathogenesis and alters the neuronal compartment of the brain. Misfolded and aggregated protein complexes bind to pattern recognition receptors on microglia and astroglia triggering an innate immune response (131, 141, 169-174). This response results in the release of inflammatory factors that increase disease severity. High throughput screening studies have revealed that many genes that increase the risk for sporadic AD are actually encoded in glial cells. Other external factors like obesity, metabolic disorders, exposure to toxins, stress and high cholesterol all indicate interference with the immunological processes of the brain. Our ability to understand and control such immune reactions in the brain would be an important step to prevent or delay neurodegenerative diseases (69, 74, 87, 94, 141, 169-171, 175-178).

The innate immune response toward amyloid deposition and neurofibrillary tangle formation is considered a prime pathological event in AD. It has been observed that in AD brains amyloid plaques are always surrounded by activated microglial cells and astrocytes. The complement-mediated immune system has also been found to be activated in amyloid- and tau tangles-positive AD brains (78, 141, 179-181). Once the amyloid plaques or tangles are formed, the immune system mediates a local inflammatory response that directs the immune cells to the site of deposition and promotes secondary inflammation. Immune-based therapies have been designed to remove amyloid peptide aggregates from the brain of AD animal models. Several generic non-steroidal anti-inflammatory drugs have been shown to be slightly effective against

AD progression (100, 182-184). The nonsteroidal anti-inflammatory drug (NSAID) R-flurbiprofen was used in a recent Phase II clinical trial in patients affected with mild AD and showed a 62% decline in AD symptoms. These NSAID's mainly target cyclooxygenase 2 (COX2), which is found to be up regulated in AD brains and enhances neuroinflammation (182, 185-188). NSAIDs such as ibuprofen and indomethacin target cyclooxygenases nonselectively and suppress prostaglandin production by microglial cells during inflammatory reactions (189, 190). Few agents directly target β-secretase and APP-CTF-β thereby hindering proteolysis of APP and APP-CTF-β respectively. In the brain, the resident immune cells sense the alterations in the tissue through receptors for damage associated molecular patterns (DAMPs) (191). In this case, DAMPs are amyloid plaques, tau fibrillary tangles and misfolded proteins. The extreme inflammatory condition observed in AD is driven mainly by CNS-resident immune cells —microglia, perivascular myeloid cells and astrocytes.

1.7 **Astrocytes- Activation and Inflammation**

Astrocytes and microglia are the two major glial cell populations of the brain. They both communicate with neurons to maintain homeostasis and proper functioning of the brain. In addition to participating in the immune response, astrocytes recycle glutamate, and regulate CNS blood flow, blood brain barrier formation and maintenance, synaptogenesis, myelination, metabolism, and release of transmitters (64, 74, 97, 141, 192-195). They are the predominant cells in the mammalian brain. Their role has been documented in several neuro-developmental diseases such as Rett Syndrome and Fragile X Syndrome and in

neurodegenrative disorders such as multiple sclerosis (MS), Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and HD (192, 196-199). Developmental studies have suggested that astrocytes develop by gliogenesis, which occurs after neurogenesis. Although it is not well understood how astrocytes develop from neural stem cells, it is believed that they follow a similar pattern as neurons. Astrocyte precursors are formed and migrate from stem cells. A maturing postnatal astrocyte is formed, from which an adult astrocyte develops. Local conditions and patterns help them develop heterogeneity (174, 192, 200-205).

Normal aging is known to be accompanied by mild inflammation, reactive gliosis and oxidative stress. In AD, astrocytes show some effects similar to microglia, but also have beneficial properties. They help in removing Aβ without the release of excessive opsonins or cytokines. After activation, astrocytes also release helpful growth factors such as nerve growth factor (NGF), S100β (neurotrophic signaling molecule), brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) (206-208). Hence they help other immune cells as well as neurons to control the release of toxic substances under inflammatory conditions. Astrogliosis is an event observed in AD brain, represented by proliferation of astrocytes in response to neuronal cell death and Aβ stimulation. Chronic inflammation in AD leads to an increase in chemokines like IL-6, IL-1β, IFN-γ which further secretes other proinflammatory cytokines (193, 209, 210).

1.8 **Microglia – Macrophages of the brain**

Microglia are the tissue-resident macrophages of the brain. They are the first line of defense for the brain against any insult or threat. The four most important functions of microglia are immune surveillance, apoptotic clearance, neurogenesis and synaptic pruning. MG share a common myeloid origin with macrophages, but have a different and distinct morphology and function (194, 211, 212). These cells develop in the bone marrow and then migrate to the brain and then adapt to their residing locations. Studies in mice have revealed that microglia originate in the yolk sac in a pU1-dependent and Myb-independent manner from erythromyeloid progenitors (EMPs). During development of the zebra fish brain, the precursor cells spread within the embryo and then, 48 hrs post fertilization, invade the brain and establish a microglial population (211-216). In neurodegenerative diseases like AD, patients suffer from microglial over activation and blood brain barrier defects arising from chronic inflammation. It is believed that microglia poorly phagocytose amyloid plaques and promote AD pathology by releasing proinflammatory cytokines. MG develop a ramified and activated phenotype around Aβ amyloid plaques in AD and lose their intrinsic beneficial role during the disease progression. Instead they acquire a toxic phenotype which is evident from the increasing Aβ build up and increase in inflammation over time (141, 170, 174, 211, 217-221).

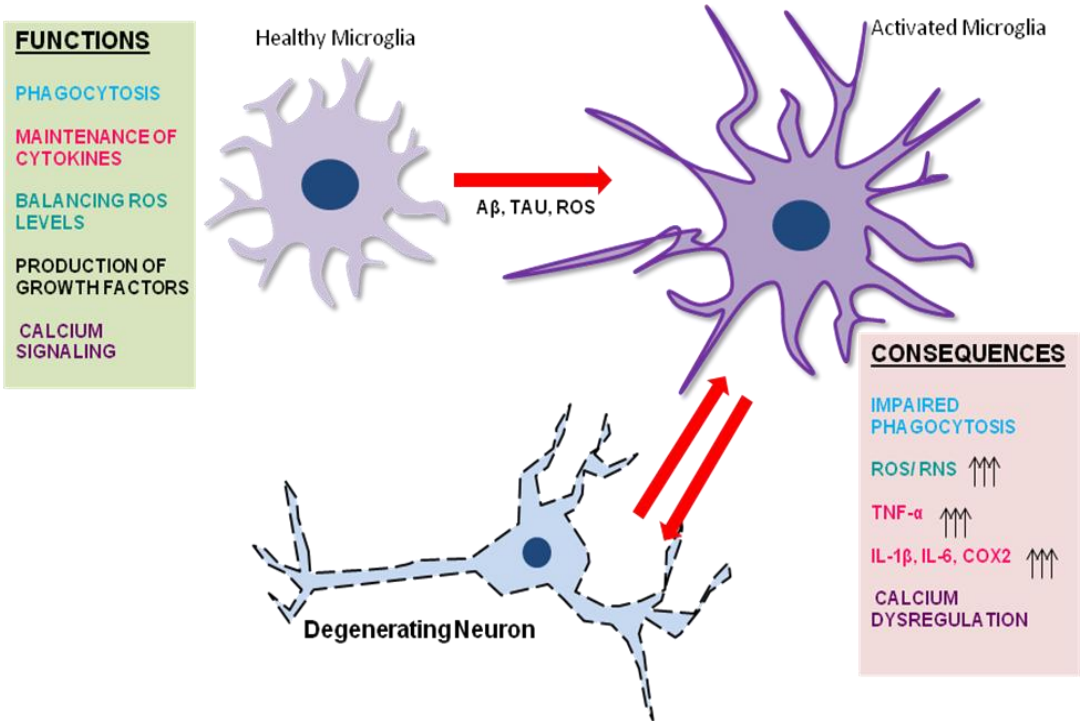

Figure 1.3 Schematic chart diagram of microglial cell in AD

Microglia are the prime immune cells in the brain. They constantly survey their surroundings for the presence of pathogens and cellular debris. It is also responsible for maintaining the homeostasis of the brain. When microglial cells encounters misfolded or aggregated proteins like Abeta or tau in AD, it triggers an innate immune response characterised by release of inflammatory chemokines and ROS, which ultimately leads to neuronal cells death.

1.9 **Various functions of Microglia**

There are several important functions of microglia in the context of brain development, homeostasis and neuronal connectivity. These different functions are described below.

1.9.1 Phagocytosis of debris and foreign bodies.

Phagocytosis is the most important and basic function of microglial cells. These are the cells that attack any kind of foreign bodies or debris found in healthy brain. The MG release multiple soluble cytotoxins, neurotrophins and immunomodulatory factors that help in clearing debris by phagocytosis. Chemokines released by degenerated neurons also act as a chemo-attractant for MG to detect their site of action. Studies have revealed that MG are highly motile at any stage and have filopodia-like protrusion used for continuous surveillance of the microenvironment (222-224). In a healthy brain, to facilitate successful repair of the microenvironment, MG create an environment necessary for regeneration. In order to do so, they release numerous cytokines, ROS/RNS, chemokines and other soluble mediators at the spot of repair. These chemicals attract phagocytotic and repair helper cells, which in turn contribute to tissue repair. This kind of repair activity has been observed in remyelination of damaged axons (131, 172, 174, 225, 226).

For the purpose of phagocytosis, MG express two major classes of receptors, toll like receptors (TLRs) and receptors that recognize phosphatidylserine (PS). TLRs are mainly responsible for phagocytosis of pathogens and any unnatural bodies in the brain (227-230). This process leads also to pro-inflammatory responses and the release of

tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), NO and ROS. Other groups of phagocytotic receptors in MG include scavenger receptors, RAGE, Scara receptors, Fcɣ receptors and integrins (54, 148, 151, 225, 231-237). These receptors are discussed in details later on.

1.9.2 Engulfment of apoptotic cells.

Apoptosis or programmed cell death is an essential step in brain development. Microglia play a significant role in removing apoptotic neurons during CNS development and in the adult brain. Studies suggest that in brain, apoptotic cells express "eat-me" signals that allow MG to recognize these cells and eventually remove them. Phosphatidylserine (238-240) (PS) is such a signal and binds PS receptors including milk fat globule epidermal growth factor 8 (MFG-E8) present on the microglial surface. The level of expression of PS determines the phagocytic behavior of MG. In zebrafish, it has been observed that brain-specific angiogenesis inhibitor 1 (BAI1) and T-cell immunoglobulin mucin receptor 4 (TIM4) proteins are required by microglial cells to perform phagocytosis of apoptotic cells. BIA1 promotes phagosomal cup formation whereas TIM4 helps in maintaining the structure of the phagosomes (241). Other signals like adenosine tri-phosphate (ATP) or lysophosphatidylcholine attract the microglia toward apoptotic neurons (241). In addition, apoptotic neurons release the chemokine fractalkine (CX3CL1) into the environment to send a "find-me" signal for the microglia (241). Another interesting signal that helps microglia in finding apoptotic cells are generated due to changes in Ca^{2+}. At the site of injury, these spike Ca^{2+} signals lead to the release of ATP (80, 242). The resulting ATP gradient activates the $P2y_{12}$ purinergic receptor (243, 244) on microglial cells, attracting them to the site of the injured neuron. MG also are

known to trigger neuronal cell death during cerebellum and hippocampus development. This process is known as "phagoptosis" and it involves the killing of Punkinje neurons by production of reactive oxygen species and superoxide anions. These ROS are produced when CD11b integrin and DAP12 are activated in MG. Studies have also revealed that release of TNF-α in activated microglia hastens the process of phagoptosis (245, 246).

1.9.3 Participation in neurogenesis and brain wiring.

MG had been considered non-functional during neurogenesis for decades. Recently, studies have revealed that they actually play an important role in neurogenesis. MG produce or support the production of neurotrophic factors such as insulin growth factor 1 (IGF-1) to promote nerve growth and survival during neurogenesis (247). Interestingly, different signaling molecules are released by microglia during development based on their location. For example in layer V, the IGF-1 signaling pathway is activated by MG whereas in the subventricular zone, interleukin 1 beta (IL-1β) and interferon gamma (IFNɣ) pathways are activated (169, 248-255). MG promote wiring of the brain, specifically at the regions close to dopaminergic and neocortical interneurons. Apart from a direct role in neurogenesis, some location specific MG also impact proliferation of neuronal precursor cells (NPC). The activation of MG inversely correlates with the number of NPC and directly impacts glial proliferation. Hence the fewer activated MG there are, the more NPC are found in the CNS (256-260).

1.9.4 Maintenance of synaptic pruning.

Synaptic pruning is one of most interesting functions of MG. Synaptic pruning is a process where synapses are remodeled during early and late postnatal stages of neuronal connections. In postnatal mouse brains, microglial synaptic pruning is observed in

hippocampus, cerebral cortex and thalamus (261, 262). The complement system, which is considered an integral part of the infection fighting process, plays an important role in brain wiring and synaptic pruning. C1q and C3 complement receptors in particular are involved in synaptic pruning through the removal of synapses through phagocytosis. It is suggested that neuronal activity is continuously sensed by microglia in the brain (263-266).

1.10 **Microglial cells in Alzheimer's disease**

AD is strongly associated with a state of chronic inflammation in the brain, a process in which MG play a significant role. The role of MG in AD became clearer when *TREM2* (gene encoding the TREM2 receptor) and the gene coding for its associated protein, TYROBP/DAP12, were found to be directly associated with late onset Alzheimer's disease through GWAS studies. TREM2 is a major phagocytotic receptor in MG. TREM2 is essentially required for phagocytosis of debris and Aβ plaques in AD. It also controls the inflammatory response of MG and helps in promoting survival and repair pathways in AD (18, 19, 226, 231, 267, 268). In addition, mutations in the microglial gene colony stimulating factor 1 receptor (CSF1R) play an important role in neurodegeneration. CSF1R is required for normal growth of resident microglia in developing brain. In EAD, CSF1R helps in microglial activation that promotes phagocytosis of Aβ plaques and TAU fibrils. Interestingly, in LAD CSF1R upregulation promotes over-activation of MG leading to an increase in inflammation (269, 270).

In AD, aggregation of beta amyloid plaques and tau fibrillary tangles within cells as well as in extracellular regions leads to over activation of microglia and neuronal cell death. Evidence suggests that Aβ fibrils attract MG. MG elevate expression of MHC class II, a sign of activation. In vivo studies in AD transgenic mice have shown that when MG come in contact with Aβ plaques and tau tangles they release numerous immune molecules including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, IL-12, IL-23 and complement proteins. In vitro studies have implicated additional cytokines and chemokines including tumor necrosis factor (TNF-α, IL-1b, IL-6, macrophage inflammatory protein (MIP)1a, macrophage chemotactic protein (MCP)-1, IL-8, macrophage-colony stimulating factor (M-CSF) and IL-12 as factors released by activated MG. Activation of the complement cascade (C1qB, C3 and C4) is also observed. Microglial specific receptors CD14, CD36, CD47, α6β1, class A scavenger receptor, TLRs and receptor for advanced glycation endproducts (RAGE) are involved in the initiation of these inflammatory pathways. All of these factors in a combined manner contribute to neuroinflammation in AD (56, 91, 148, 172, 209, 225, 248, 271-276). Complexities arise when we try to define the role of such neuroinflammation in AD brain. Evidence suggests that this inflammatory response is beneficial to the brain in the early stages of disease progression, but prolonged chronic inflammation forces MG to attain an altered state that causes more harm than good. MG at this stage continue secreting neurotoxic chemokines, cytokines, ROS, RNS and superoxide ions which worsens the AD pathology by increasing stress in the microenvironment (75, 141, 177, 178, 182, 217, 277-281).

Like other cells of myeloid origin, MG perform phagocytosis with high efficiency in healthy brain. In AD brain, MG are supposed to phagocytose Aβ and degrade Aβ by microglial proteases, which are key mechanisms for removal of these aggregates from the brain. Studies have indicated that in moderate or later severe stages of AD, the phagocytosis of Aβ plaques and fibrils by MG is impaired. The increasing burden of Aβ plaques in the AD brain increase chronic inflammation and leads to cellular senescence (219, 282, 283). Senescence in MG results in a state where MG cannot phagocytose but generates pro inflammatory factors, cytokines and reactive species. Soluble Aβ (sAβ) has been reported to be engulfed by MG through both phagocytosis and pinocytosis. Pinocytosis is an endocytic process where MG uptake soluble Aβ by forming an invagination. The Aβ is then suspended in small hollow vesicles. The content of these vesicles are further hydrolyzed in lysosomes and degraded (284, 285).

Genetic mutations in microglia have been found to promote AD pathogenesis. For example, the TREM2 adaptor molecule, TYROB/DAP12 is associated in LOAD pathogenesis. TREM2 is one of the major receptors required by MG for phagocytosis of debris and foreign bodies. DNAX-Activation Protein 12 (DAP12) is an adaptor receptor protein that helps in ITAM receptor signaling in MG. It also interacts with CSF1R in Hereditary Diffuse Leukoencephalopathy with Spheroids (HDLS) pathology (16, 21, 231, 286, 287). Genome wide association studies (GWAS) studies revealed an increase in microglia of CD33 (Siglec-3) in human AD brain, which is correlated with increased insoluble Aβ levels. CD33 receptors bear an ITIM that is required for receptor signaling in MG. Increase in CD33 activity in AD mouse models revealed reduced uptake Aβ clearance by MG. This suggests that CD33 activity inhibits phagocytosis ability of MG

through ITIM receptor signaling (14, 288-290). TREM2 on the other hand is involved in cytokine and chemokine production, phagocytosis and MG cell motility. These studies on microglial specific genes have provided us with deeper insight in understanding the role of MG in AD progression and specifically, sporadic AD.

1.11 **SYK kinase and its activation**

SYK is a non-receptor protein-tyrosine kinase located in the cytoplasm. SYK was initially discovered as a 40 kDa catalytic fragment by two independent groups as published in Zioncheck et al. and Kobayashi et. al. Later, the full-length72 KDa enzyme was identified through the use of specific antibodies (291, 292). SYK is mainly expressed in spleen and thymus and is found in many hematopoietic cells including B cells, thymocytes, erythrocytes, macrophages and platelets (271, 293, 294). SYK also has been found in a subset of non-hematopoietic cells including epithelial, endothelial, fibroblast, neuronal cells and some non-neuronal brain cells (295, 296). SYK is well known to play crucial roles in adaptive immune receptor signaling, innate immune signaling, platelet activation, cellular adhesion, cellular motility, vascular development, epithelial cell division, and osteoclast maturation (297, 298). SYK function has been linked to several disorders including autoimmune diseases, hematological malignancies, rheumatoid arthritis and inflammation (299-306).

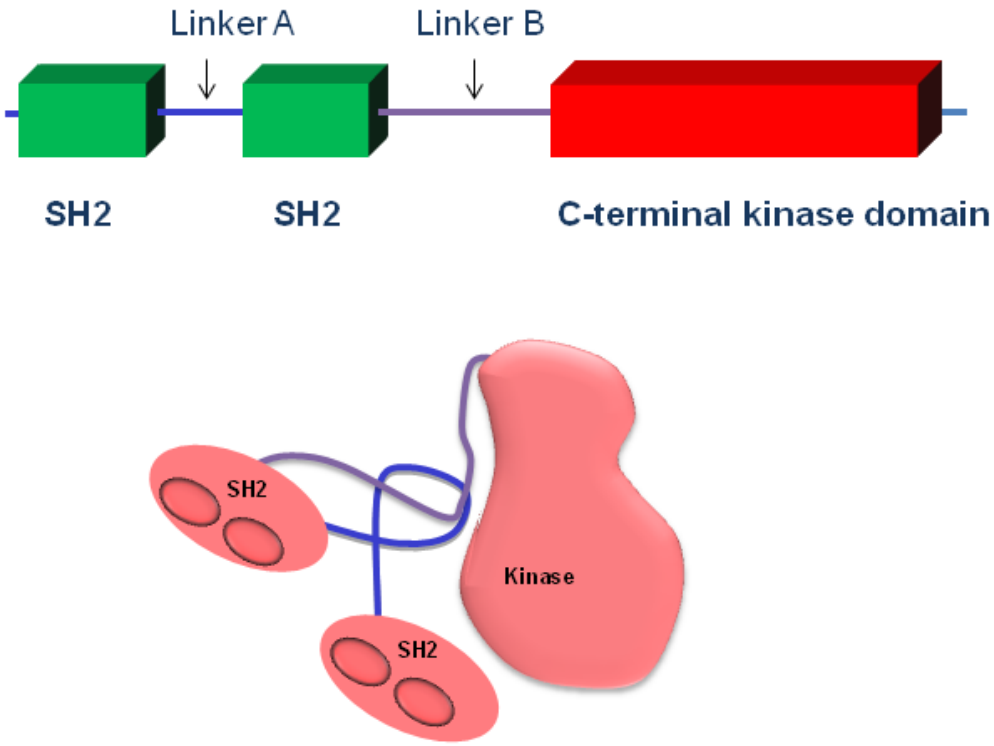

Figure 1.4 Schematic diagram of SYK structure

SYK is a protein tyrosine kinase which consists of a C-terminus catalytic domain to promote activation of downstream proteins. It also has two tandem SH2 domains at the N-terminal that helps in receptor signaling through ITAM binding.

As a kinase, SYK phosphorylates multiple proteins on tyrosine to control key receptor signaling pathways including Ca^{2+} mobilization and the phosphoinositide 3-kinase (PI3K)/AKT, NF-κB and RAS/ extracellular-signal-regulated kinases (ERK) pathways (307-313). At least two substrates of Syk, CD79a and the Ikaros protein, also have been reported to be phosphorylated by SYK on serine, suggesting that SYK may have capabilities beyond catalyzing tyrosine phosphorylation. Its ability to couple with immune recognition receptors makes it an important kinase in the immune system that regulates multiple pathways in response to many extracellular signals including antigens and immunoglobulin-antigen complexes (314).

The N-terminus of SYK consists of two 100 amino acid conserved sequences known as Src Homology 2 (SH2) domains. These SH2 domains are connected by a region known as linker A and both are then separated by linker B from the C-terminal kinase domain. These domains bind to proteins containing phosphotyrosine and thus initiate protein-protein interactions. The two SH2 domains of SYK bind specifically to proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs). ITAMs are characterized by two short peptide sequences of YXXL/I (where Y is tyrosine, L and I are leucine and isoleucine, respectively, and X denotes any amino acid) separated by a spacer of 6-12 amino acids. Once receptors with ITAMs are oligomerized upon engagement, the two tyrosines are rapidly phosphorylated leading to the recruitment and activation of SYK (23, 291, 304, 315-317). In some cells, particularly T cells, the SYK-family kinase ζ-chain-associated protein kinase of 70 kDa (ZAP70) is recruited to phosphorylated ITAMs (318, 319). Following its activation, multiple tyrosines on SYK become phosphorylated including sites within the linker A and B regions along with

tyrosines within the catalytic domain and at the extreme C-terminus. SYK both autophosphorylates and is phosphorylated in *trans* by LYN. Examples of important phosphorylation sites include Y130 located in linker A, Y317, Y342 and Y346 located in linker B, Y519 and Y520 located on the activation loop in the catalytic domain and Y624 and Y625 near the C-terminus. Phosphorylations serve to regulate the activity of SYK, to modulate kinase-receptor interactions and to promote protein-protein interactions with downstream effectors containing SH2 or related domains (304, 320, 321).

Following its activation, a fraction of SYK is released from the receptor and can shuttle between the nucleus and the cytoplasm to promote further downstream signaling events. Included among the SYK substrates that have been well characterized are the B cell linker protein (BLNK/SLP65), SH2 domain leukocyte-specific phosphoprotein 75 kDa (SLP76) , linker for activation of T cells (LAT), PI3K, phospholipase Cɣ (PLCɣ), Bruton's tyrosine kinase (BTK), Vav-1 (Ras/Rho guanine nucleotide exchange factor), β-tubulin, and CBL (E3 ubiquitin ligase) (322-330).

1.12. **Receptor based immune signaling of SYK**

1.12.1 Syk in B cell signaling.

A major component of the adaptive immune response in the body requires B cell activation for producing antigen specific antibodies to target foreign molecules and pathogens. It is the B cell receptor for antigen (BCR) that recognizes foreign antigens. The BCR consists of a membrane-bound immunoglobulin associated with a heterodimer of CD79a and CD79b subunits. CD79a and CD79b also are commonly known as Ig-α

and Ig-β. The BCR contains ITAM sequences in the cytoplasmic tails of CD79a and CD79b that are initially phosphorylated by a member of the Src-family of protein-tyrosine kinases. The main Src-family kinase in B cells is LYN, which phosphorylates the first tyrosine of the ITAM. The second tyrosine is speculated to be phosphorylated by SYK based on in vitro studies. Once both of the ITAM tyrosines are phosphorylated, SYK binds tightly through its tandem SH2 domains. Once bound to the BCR, active Syk phosphorylates additional ITAM tyrosines leading to additional SYK binding to the BCR complex. Activated SYK then catalyzes the phosphorylation of the multiple protein substrates that play important roles in antigen receptor signaling to regulate downstream cell signaling pathways. These include for example, the adapter protein BLNK, whose phosphorylation creates docking sites for BTK and PLC-ɣ to form a complex ultimately facilitating calcium mobilization (315, 331-336).

1.12.2 Syk in mast cell signaling.

Mast cells and basophils are key players in inflammation. Their activation results in type I allergic reactions through the release of pre-formed inflammatory mediators like histamine from cytoplasmic granules and the generation of additional biologically active mediators like leukotriences. Mast cells are enriched with high affinity IgE receptors (FcεR1) on their cell surface (23, 291, 317, 337-340). The stimulation of mast cells is initiated by the binding of antigens to FcεR1 receptors pre-associated with IgE. FcεR1 is a tetrameric protein consisting of $\alpha\beta\gamma_2$ chains. The Fc region of IgE binds to the α-chain of IgE. ITAMs are present in the β- and ɣ- chain, located in their cytoplasmic domains. Similar to the BCR, FcεR1 does not have an intrinsic enzymatic activity, but relies on the activation of non-receptor protein-tyrosine kinases for its activity (311, 341-343).

LYN initiates the phosphorylation of the tyrosines on these ITAMs leading to the docking of SYK and its activation. Active SYK further propagates downstream tyrosine phosphorylation of adaptor proteins such as LAT1, LAT2 and SLP76. Several other substrates including PI3K, BTK, PLCγ1, PLCγ2 and PKC are subsequently activated through this pathway. Studies indicate that the absence of SYK in mast cells leads to a complete loss of IgE-mediated degranulation and release of cytokines, making SYK a key mediator of mast cell signaling and a popular drug target (297, 305, 317, 344, 345).

1.12.2 Syk in macrophage signaling.

Macrophages are crucial components of the innate immune response and are the first line of defense against pathogens. The most important function of macrophages is to recognize foreign substances to either engulf and degrade them or to engage other immune cells to destroy them. They are white blood cells of the myeloid lineage and differentiate in tissues from circulating monocytes. During inflammation, macrophages perform vital functions including phagocytosis, antigen presentation and immunomodulation (271, 346-349). The activation of macrophages occurs through various stimuli including cell surface components of pathogens such as lipopolysaccharide (LPS), cytokines such as interferon-γ and TNF-α, apoptotic cells and TH1 T cells. Macrophages express a broad range of receptors including the toll-like and dectin-1 pattern recognition receptors, scavenger receptors, and Fcγ receptors that recognize foreign substances and opsonized particles in the body (125, 350, 351). Many of these receptors are coupled to downstream signaling pathways through protein kinases that, in turn, activate transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and cAMP response element binding protein (CREB) that promote the

expression of proinflammatory genes. Many of these receptors also trigger phagocytosis, the secretion of inflammatory mediators, the generation of ROS, the production of nitric oxide and the generation of arachidonic acid metabolites such as prostaglandin E_2 (PGE_2) (137, 337, 352-361).

Interestingly, many of these receptors in macrophages contain ITAM sequences and are thus dependent on SYK for their activities. These include the Fcɣ receptors, which are mainly involved in the phagocytosis of foreign bodies to which IgG is associated. These belong to the immunoglobulin gene superfamily. Fcɣ receptors are classified into three categories: class I and III, which form multimeric complexes, and class II receptors that are monomeric. All Fcɣ receptors contain an extracellular binding domain that recognizes the Fc region of IgG. Macrophages primarily express activating Fcɣ receptors that stimulate phagocytosis and other innate immune responses. Clustering of these receptors leads to the Src-family kinase-mediated phosphorylation of tyrosines within ITAMs found either within the receptor or receptor complex. Subsequently SYK is recruited to the phosphorylated ITAMs and activated, resulting in the phosphorylation of other Fcɣ receptor ITAMs and downstream substrates. FcɣRI, FcɣRIIA, and FcɣRIIIA receptors specifically bind to SYK and their clustering leads to its activation. Several studies in primary macrophages have revealed that the absence of SYK leads to a failure of Fcɣ receptor-mediated phagocytosis and a loss of the receptor stimulated generation of NO and ROS, events that are normally enhanced by Fcɣ receptor engagement in macrophages (25, 26, 292, 315, 362-364).

LPS is a commonly used stimulus for macrophages. LPS is found on the surfaces of Gram-negative bacteria. Thus, exposure to LPS mimics a bacterial infection. The

TLR4 receptor is needed for macrophages to respond to LPS. Interestingly, SYK also is activated in macrophages by LPS stimulation. There is evidence that SYK is constitutively docked to TLR4 receptors despite the lack of an ITAM. Active Syk thereby promotes activation of downstream signaling molecules, such as AKT, PI3K, IKK, NF-κB and phosphoinositide-dependent kinase-1 (PDK1), ultimately leading to induction of TNF-α, COX-2 and inducible nitric oxide synthase (iNOS), and the secretion of nitric oxide (NO), ROS and PGE_2. Studies also indicate that minimally oxidized low-density lipoprotein (mmLDL) activates SYK in macrophages throughTLR4. Activated SYK subsequently activates Vav1, PLCɣ and JNK resulting in cytoskeleton modifications, ROS generation and cytokine secretion (293, 312, 365-369).

1.12.4 Syk in neutrophil signaling.

Neutrophils are also essential cells in the innate immune system. They are the most abundant type of white blood cell and also the first cell type to reach a site of inflammation. Once in contact with cellular and humoral factors, they become primed and travel through blood vessels to the site of microbial infection. Their main function is to kill or suppress microorganisms during the innate immune response.

Integrin receptors are particularly important receptors in neutrophils and are required for cell adhesion, motility, spreading, the respiratory or oxidative burst, release of cytokines and release of antimicrobial granule proteins. β_1, β_2, and β_3 integrins in neutrophils all associate with SYK to mediate their functions. Neutrophils mainly express the β_2 integrins $\alpha_L\beta_2$, $\alpha_M\beta_2$ and $\alpha_4\beta_1$. Integrin signaling occurs in two steps, known as inside-out (integrin activation) and outside-in (aggregation of integrins) signaling. SYK is essentially involved on both these signaling pathways. Studies in SYK negative

neutrophils have revealed that, in the absence of SYK, neutrophils fail to spread when plated on integrin ligands and fail to undergo respiratory bursts or degranulation in response to integrin clustering. Integrins themselves lack ITAM motifs, but in neutrophils signal instead through the adaptor proteins DAP12 and FCɣR, both of which contain ITAMs that, when phosphorylated, bind and activate SYK (246, 286, 303, 327, 370-372).

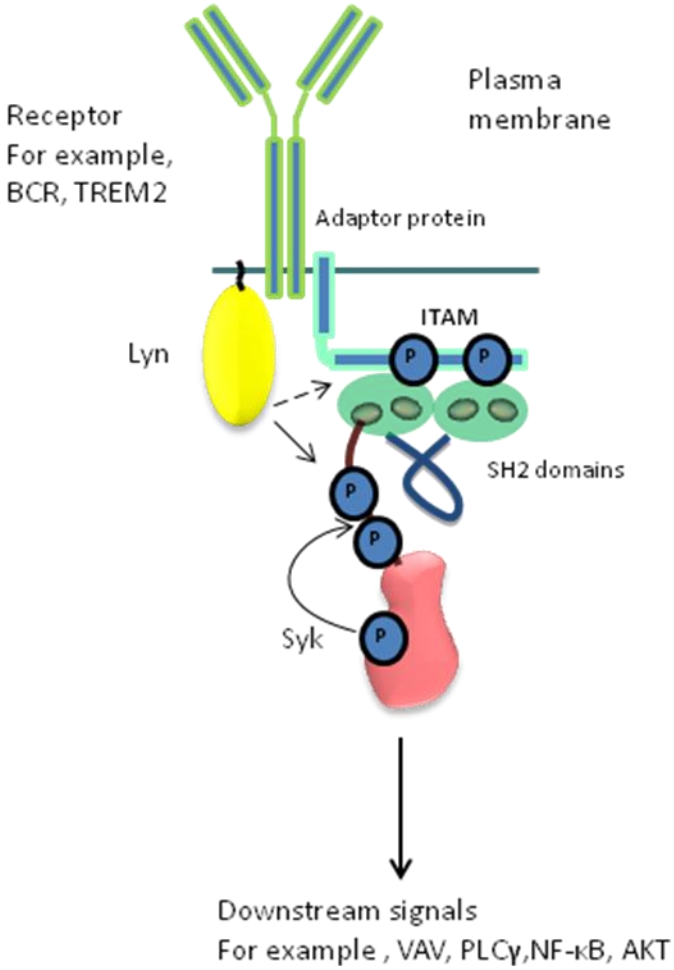

Figure 1.5 Schematic diagram of adaptor mediated SYK-ITAM signaling

SYK is activated through binding of receptor ITAM to SH2 domains. In receptors like BCR, it requires LYN kinase to initially phosphorylate ITAM on receptor adaptor molecule. Once activated the receptor further activates through autophosphorylation and initiation of SYK-ITAM cascade. This activation leads to activation of several downstream signaling pathways through tyrosine phosphorylation.

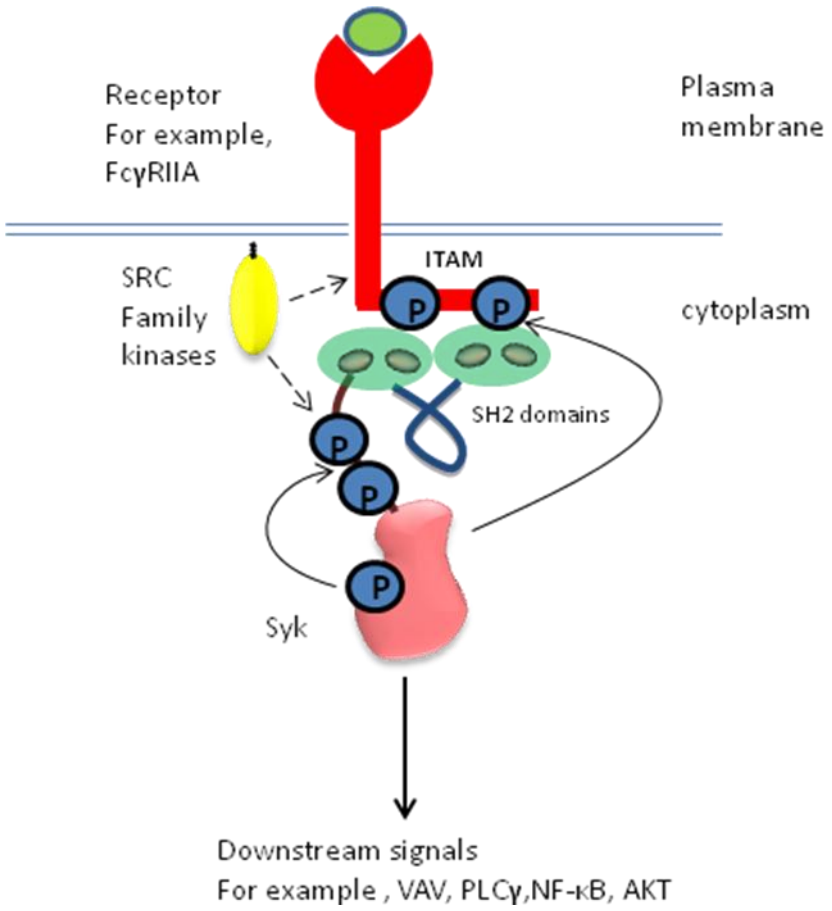

Figure 1.6 Schematic diagram of direct ITAM-SYK signaling

In some rectors like FcɣRIIA, SYK is activated through binding of receptor ITAM present at the cytoplasmic tail of the receptor. It does not require an adaptor protein for activation. LYN kinase initially phosphorylates ITAM on receptor tail and SYK binds to phosphorylated ITAM through SH2 domains. Thus SYK-ITAM signaling cascade is initiated.

1.13 **Syk in the brain**

Brain cells can be classified into neuronal and non-neuronal cells. Non-neuronal cells in the brain include glial cells and other helper cells that assist with the normal functioning of the brain. Glial cells mainly consist of astrocytes, oligodendrocytes and microglia. One crucial function of glial cells is to comprise the immune system of the brain, which functions quite differently from the rest of the body. The presence of the blood brain barrier restricts the ability of normal immune cells in the blood to infiltrate into a healthy brain to maintain immunity. While the brain had long been considered a completely immunologically privileged site, interesting new data indicate the presence of a lymphatic system that allows T cells access to the meninges (259, 287, 311, 369, 373-375).

Although the role of SYK in the brain is poorly understood, considering its vital role in the majority of immune cells; SYK's role in the brain's immune system cannot be neglected.

1.13.1 Syk in neurons

Few in vitro studies have suggested the presence of SYK in neurons. It has been mentioned that SYK is involved in neuronal differentiation in P19 cells (376). Another study on the CD3ζ adaptor immune protein in the CNS has suggested that SYK is involved in neuronal morphogenesis (377). The presence of an ITAM on CD3ζ allows it to bind to the SYK SH2 domains thereby activating SYK. Without SYK, CD3ζ could not promote neuronal morphogenesis. Studies on sensory neurons have reported that SYK is required for NF-κB activation through the cytokine ciliary neurotrophic factor (CNTF).

Upon CNTF stimulation, SYK is activated and phosphorylates IκB leading to NF-κB activation. This pathway also promotes neurite growth from developing neurons (313, 378).

1.13.2 Syk in microglia

Microglia are the most vital immune cells in the brain. In the CNS, they are mainly responsible for phagocytosis of debris, foreign particles, apoptotic cells, and microbes. Apart from these activities, they also maintain homeostasis in the brain through the secretion of anti-inflammatory and pro-inflammatory factors. Phagocytosis by MG is mainly mediated through various receptor types including TLRs, PTRs, PAMPs, RAGE, SCARA1, SCARA2, CD36, CD68, integrins and Fcγ receptors. Many of these immune receptors contain ITAMs as discussed above in macrophages (141, 148, 225, 228, 231, 272, 379-381).

ITAM-mediated receptor signaling through SYK is crucial for phagocytosis. DRAPER, for example, is a phagocytotic receptor in *Drosophila* that binds to Shark (fruit fly version of SYK) and is important for phagocytosis. Both phosphorylation of DRAPER ITAM tyrosines and SHARK activation are required for proper phagocytosis of damaged axons and debris (259, 382). The homologs of DRAPER in mammals are JEDI1 and multiple epidermal growth factor-like domains 10 (MEGF10). In the peripheral nervous system (PNS), 50% of the neurons are eliminated through apoptosis during development. JEDI1 and MEGF10 are essential for glial cell precursors to mediate phagocytosis of apoptotic neurons during development. Both contain two ITAMs in their intracellular domains that, when phosphorylated, bind to the SH2 domains of

SYK. In the absence of SYK, engulfment of apoptotic neurons is inhibited, but phagocytic activity can be recovered when cells are reconstituted with SYK (259).

DAP12/TYROBP is an adapter protein that contains an ITAM and is important for phagocytosis by mammalian MG. DAP12 associates with both TREM2 and signal regulatory protein-β1 (SIRPβ1). When phosphorylated following receptor engagement, DAP12 associates with SYK to mediate phagocytosis of damaged neurons, demyelinated axons and other cellular debris. This involves the binding of the SH2 domains of SYK to the phosphorylated ITAM of DAP12. TREM2 is important for the uptake of apoptotic neuronal cells. Additional studies suggest that TREM2 also binds to lipo-oligosaccharides of both Gram-positive and -negative bacteria. Binding stimulates TREM2 and, via ITAM and SYK-dependent signaling, promotes phagocytosis of bacteria. Interestingly, certain heterozygous mutations in TREM2 are correlated with enhanced incidence of AD. Furthermore, DAP12 was identified recently as a key regulator of genes associated with late-onset AD (18, 21, 231, 254, 287, 383, 384).

Immunoreceptor tyrosine based inhibition motifs (ITIMs) resemble one-half of an ITAM. Receptors bearing ITIMs negatively regulate phagocytosis. ITIMs inhibit ITAM-mediated signaling by recruiting the SH2 domain-bearing protein tyrosine phosphate SHP1 and the lipid phosphatase SHIP1 to counteract the activation of protein and lipid kinases that occurs during ITAM-mediated signaling (21, 385). CD33 related sialic acid-binding immunoglobulin superfamily lectins (SIGLECS) are examples of proteins bearing ITIMs. They belong to the Ig superfamily and recognize specific sugar residues on cell surface glycans. Since they contain an ITIM sequence in their cytoplasmic domains, they negatively regulate ITAM-SYK receptor signaling in MG. Interestingly,

CD33 expression is enhanced in AD brains and has been linked genetically to AD in GWAS studies (20, 288, 289, 386).

Another group of receptors that play a vital role in the phagocytotic ability of MG are Fcɣ receptors. As discussed above, many of these contain ITAMs and thus require ITAM-SYK mediated signaling for engulfment of opsonized particles. Clustering of Fcɣ receptors initiates ITAM phosphorylation, which in turn docks SYK through its SH2 domains. This ITAM-SYK interaction further activates SYK and hence activates downstream signaling pathways to facilitate phagocytosis of opsonized particles and antigens (9, 273, 387-389).

DECTIN-1, a major beta-glucan receptor that promotes phagocytosis of fungal pathogens, also is associated with SYK in MG. DECTIN-1 is unusual in that it's cytoplasmic tail contains only one-half of an ITAM (known as a hemITAM). DECTIN-1 is thought to function as a dimer whereby two DECTIN-1 molecules bind simultaneously to a single SYK molecule to promote its activation (223).

Several studies have revealed that SYK is essential not only for phagocytosis by MG, but also for cytokine production, NO synthesis and ROS generation. SYK mediates IL-1β and TNF-α secretion in MG through activation of NF-κB. The secretion of these cytokines into culture media can promote apoptosis of co-cultured adult neurons. β-amyloid fibrils or prion protein aggregates can activate SYK in MG cells. This activation is accompanied by intracellular calcium release and PKC activation leading to the production of IL-1β and TNF-α (210, 221).

1.14. **Stress granules and their components**

SGs are cytoplasmic RNA-protein complexes that appear in eukaryotic cells in response to various kinds of stress-related stimuli that impair translation. Some of these stressors include heat, viral infection, oxidative stress, ultraviolet irradiation, and hypoxia. SGs were first identified in cells cultured at high temperature, which led to the formation of cytoplasmic granules containing heat shock proteins. The phosphorylation of eukaryotic translation initiation factor 2-alpha (eIF2α) is one of the initial steps in SG assembly induced by stress. Based on the stress stimulus, distinct eIF2α kinases can be activated ((protein kinase R (PKR), protein kinase RNA-like endoplasmic reticulum kinase (PERK), Heme-regulated eIF2α kinase (HRI) and general control nonderepressible 2(GCN2)) (390-392). The subsequent phosphorylation of eIF2α stalls assembly of ribosomes at the 48S pre-initiation complex. As a result, this initiation complex remains assembled at the 5' end of mRNAs resulting in translation impairment and a block in polysome assembly. These nonpolysomal transcripts are then aggregated into complexes along with many other RNA binding proteins to form SGs (393, 394).

Since their discovery, numerous studies have shown that SGs contain many biological components important for mRNA metabolism. The major components include nontranslating mRNAs, translation initiation complexes, RNA binding proteins, mRNA processing helper proteins, aggregated proteins, kinases and other proteins involved in stress signaling. Some of the major proteins involved in SG formation are: AU rich element binding proteins, Argonaute, eIF2α, fragile X mental retardation protein (FMRP), fragile X mental retardation related protein 1 (FXR1), Ras-GTPase-activating protein

SH3-domain-binding protein (G3BP), glycine-tryptophan 182 (GW182), poly(A)-binding protein 1 (PABP1), T cell internal antigen-1 (TIA-1) and T cell internal antigen-1-related (TIAR) (395-398).

Recent studies have shown that many proteins responsible for RNA splicing, transcription, adhesion, immune signaling, autophagy and apoptosis also associate with SGs. The function of SG formation in various biological systems or conditions is yet to be completely deciphered. Studies suggest sequestration of mRNAs in SGs is a way to protect them from stress and conserve energy to overcome stress. SG are highly dynamic in nature and self-assemble and disassemble based on changes in the environment of the cell (28, 399).

1.14.1 SG assembly and disassembly

The presence of diverse proteins, RNAs and other biological molecules suggest that SG assembly is very dynamic and variable depending on the biological system on the type of stress induced. In some instances, composition of SGs depends on the type of mRNPs present in the SG at that specific stress stimulation. Since specific isolation of SGs has not been possible yet, most of the studies on SG are done through microscopy or correlated to the function of SG components under stress conditions. Such studies have reported that the size and shape of SG can vary in different systems. It also mentions that SGs do not confer to a definite structural organization (398, 400-403).

SG assembly is mainly modulated by three factors: covalent protein modifications, protein-protein interactions and microtubule network dynamics. In terms of protein modifications, a key process is phosphorylation. As described above, eIF2α phosphorylation is one of the most crucial steps in SG assembly. Other proteins that

undergo phosphorylation during SG assembly are TRISTETRAPROLIN (TTP), B-related factor 1 (BRF1) and Ras-GTPase-activating protein SH3-domain-binding protein (G3BP). In addition, the acetylation of histone deacetylase 6 (HDAC6) also has been reported to affect SG assembly as has protein methylation and methyl group binding to proteins with Tudor domains. One study also mentioned the role of ubiquitin-dependent proteasome system (UPS) in SG assembly. UPS interference modulates mRNA decay. It promotes phosphorylation of eIF2α and sequestration of AU-binding proteins (AUB) and ARE in SGs thereby preventing their decay under stress (15, 27, 398, 404).

Recent studies have shown that protein-protein interactions are critical for SG assembly. Many RNA-binding proteins like G3BP contain dimerization domains that promote protein-protein interactions that aid in SG formation. In fact, the overexpression of G3BP alone is sufficient to induce SGs. Additionally, RNA metabolism helper proteins like TIA-1 that contain QN-rich prion-like domains also participate in SG assembly through self-aggregation. Examples include the prion-like proteins TIA-1 and TIAR, which accumulate in the cytoplasm as complexes under SA or MG132 mediated stress promoting SG assembly. Proteins susceptible to unfolding or misfolding ((TAR DNA-binding protein 43 (TDP-43), Fused in Sarcoma (FUS) and TAU)) observed in several diseases like ALS and AD also bind to each other along with other RNPs to promote SG assembly. A third important factor that contributes to SG assembly is the microtubule network. Microtubules associated motor proteins like dynein and kinesin also associate with SGs and regulate their assembly and disassembly (398, 405-407). SGs disassemble following removal of the stress. Stress removal repairs the translation initiation machinery and helps SG components to disassociate to allow mRNAs to resume

translation or to be transferred to processing bodies for degradation. Heat shock proteins that disaggregate prion like-proteins have been correlated with SG disassembly. The RNA-binding protein Staufen1 (STAU1) has been associated with SG dynamics. The overexpression of STAU1 promotes SG disassembly whereas knockdown promotes assembly. Studies suggest that STAU1 promotes the stabilization of the mRNA-polysome complex during this process (408). Another study showed that, upon stress removal, FAK promotes the phosphorylation of growth factor receptor-bound protein 7 (GRB7) to weaken its interactions with SG component proteins like TIA-1, leading to SG disassembly (409).

Autophagy has been recently linked to SG removal. It has been shown that SG clearance is reduced in eukaryotic cells by inhibition of autophagy or depletion of proteins like valosin-containing protein (VCP) and autophagy related protein (ATG) which is required for autophagy machinery in cells (32).

1.14.2 SG components

Depending on the cell type, stress induced and biological system under study, the components of SG can vary. The first class of SG components represents members of translation initiation complexes. Major proteins in this category include eIF2α, eIF3, eIF4F, eIF4B, components of small ribosomal units and PABP-1. These are considered core components of SGs. The second class of components are the mRNA binding proteins, translation modifying proteins and proteins involved in mRNA stability. Few examples of such proteins are TIA-1, TIAR, FMRP, FXR1, FAST, ARGONAUTE, cytoplasmic polyadenylation element binding protein (CPEB), ATAXIN-2, and RNA-associated protein 55 (RAP55). Proteins involved in RNA decay that are SG components

include TTP, BRF1, receptor for Activated C Kinase 1 (RACK1), Z-DNA Binding Protein 1 (ZBP1) and PMR1 (27, 28, 394, 406, 410).

Finally, the third class of SG components consists of RNA binding protein that controls RNA decay and processing. In this category we have G3BP, CAPRIN, FAST, survival of motor neuron (SMN) and Ins (1,3,4,5,6)P5 2-kinase (IP5K). Few of these SG components like TIA-1, G3BP can be a part of both second and third class. Recent studies have shown that some proteins that do not directly interact with RNA, interact instead with core SG components to become a part of a SG. For example, steroid receptor coactivator-3 (SRC-3), TAU, folate-binding protein (FBP) proteins are found in SGs because of their ability to interact with TIA-1; TNF Receptor-Associated Factor 2 (TRAF2) on the other hand binds to eIF4G and DIS1 binds to eIF3 (29, 411-413).

1.14.3 SG functions

The true function of SGs in the cell is not totally clear. Studies indicate that SGs are present in very different biological systems and participate in multiple processes. SG components have roles in biological functions like mRNA stability, mRNA translation and mRNA modification outside of their localization in SGs. Hence knockdown studies of SG components do not directly address the function of SGs. One of the major suggested consequences of SG formation is to regulate mRNA stability. Formation of SGs containing untranslated mRNAs under stress protects them from degradation in the cytoplasm. Once the stress is gone and SG disassembly occurs, these mRNAs are freed to participate normally in translation. Since SGs consist of mRNP complexes including translation initiation complexes the formation of SGs under stress may assemble and store specific mRNP proteins for participation in future translation processes. Since under

stress translation is stalled, excess mRNPs required for translation are stored as SGs which are later utilized once the stress is gone (398, 404, 414-416).

Under stress, a cell ultimately needs to decide if it will be able to recover from the stress or, if the stress is too extreme, to undergo apoptosis through programmed cell death. SGs play important roles in the apoptosis of cells under stress. For example, MAP kinase kinase kinase (MTK1) is activated in cells under severe stress. MTK1 activation-mediated apoptosis takes place through an interaction with receptor for activated C kinase 1 (RACK1). In vitro studies have shown that, under stress, RACK1 is sequestered to SGs, thereby inhibiting MTK1 activation and preventing apoptosis (417, 418).

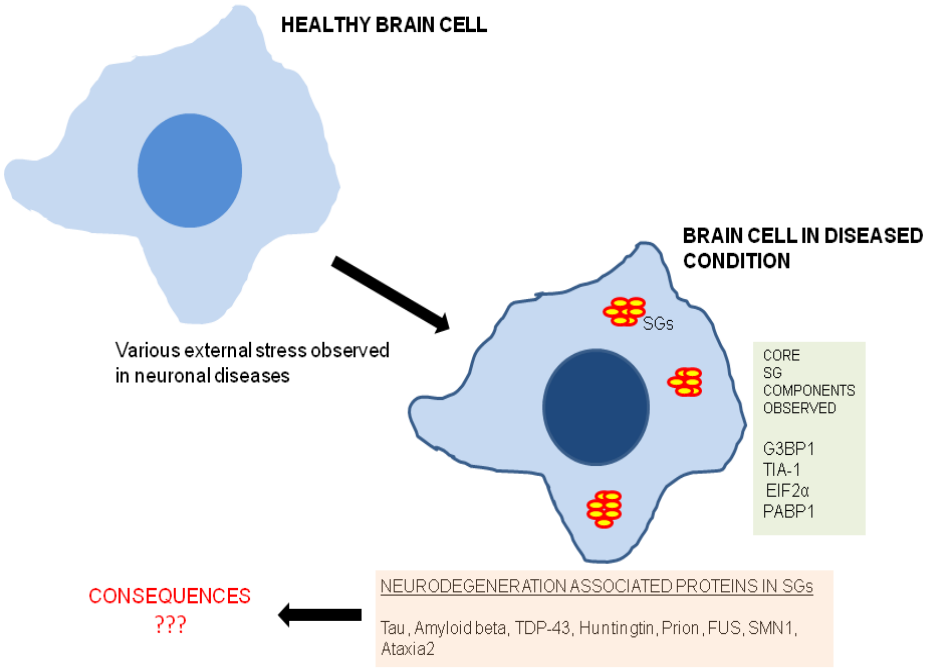

Figure 1.7 Schematic diagram of stress granules in neurodegeneration

In neurodegerative diseases like AD, ALS and SMA, presence of SGs have been reported in human brain and animals models. Core SG components like G3BP1, TIA-1 associate with misfolded and aggregated proteins like TAU, TDP-43 in neurons. The size and number of stress granules increases with the disease progression. Possible role of stress granules in neurodegeneration is yet unknown.

1.15. **Stress granules in different neurological diseases**

1.15.1 SGs in Alzheimer's disease

AD is a progressive neurological disorder. As mentioned earlier, oxidative stress is one the key players in AD. Protein misfolding and accumulation of tau proteins and Aβ is a common event in AD. As a consequence, chronic neuroinflammation and neuronal cell death is observed. Studies have suggested that SG components like TIA-1, TTP and G3BP associate with pathogenic tau epitopes. TIA-1 positively regulates apoptotic cell death and regulates mRNA translation of TNF-α and COX-2. TTP on the other hand associates with mRNA for TNF-α, GM-CSF, COX-2, IL-3, IL-10, and interferon-ɣ making it an important protein in inflammatory pathways (29, 419, 420).

1.15.2 SGs in Amyotropic Lateral Sclerosis (ALS)

ALS is a neurodegenrative disease that mainly affects the motor neurons. It is considered the most common motor neuron disease. The neuronal death mainly takes place at the brain stem, spinal cord and motor cortex. As a result of such degeneration, muscle fasciculation, wasting and weakness; increased spasticity, and hyperreflexia are observed. One of the hallmark proteins involved in ALS is TDP-43. In ALS patients, TDP-43 is mutated, mis-folded and aggregated in degenerating motor neurons. Recent studies have shown that TDP-43 is localized in SGs in ALS. Another disease that is closely linked with ALS is Frontotemporal Lobar Degeneration (FTLD). TDP-43 is also mutated in FTLD. TDP-43 is mainly a nuclear protein, but in neurodegerative diseases it is mis-localized to the cytoplasm. TDP-43 plays an important role in mRNA metabolism. In neurons, it mediates splicing and mRNA stability. Hence it influences proteins that are

required for proper formation of synapses in neurons. Since TDP-43 is localized to cytoplasmic SGs in ALS, it cannot further maintain mRNA stability. On the other hand, TDP-43 has a self aggregating domain and an excess of TDP-43 promotes aggregation. Such aggregation of TDP-43 in turn elevates its expression. Excess TDP-43 has been reported as neurotoxic to neurons and promotes apoptosis. TDP-43 tightly binds to G3BP in neurons. This binding promotes self aggregation of TDP-43 leading to larger SGs. Other proteins that localize to SGs in ALS are FUS, HUNTINGTIN and PrP (38, 405, 406, 421-423).

1.15.3 SG in spinal muscular atrophy

SMA is a recessive neuromuscular disorder that affects the α-motor neurons. It mainly occurs in infants and involves the spinal cord. Mutations in or deletions of the telomeric copy of the *SMN-1* gene and retention of the telomeric copy of *SMN-2* characterizes this disease. SMN plays a key role in axonal growth and maintenance through regulation of mRNA metabolism. SMN has been reported to be localized in SGs. SMN localization in SGs impairs its ability to regulate protein translation leading to neuronal cell death (412, 424, 425).

CHAPTER TWO: MATERIAL AND METHODS

2.1 **Cells and cell lines**

BV-2 microglial cells developed by Blasi and colleagues (387) were obtained from Dr. Chris Rochet (Purdue University) and cultured in high glucose DMEM media containing 10% FBS and 100 U/ml penicillin and streptomycin. The cells were passaged at a ratio of 1:8 every 48 h and a confluency of 60-80% was maintained at all times. Fresh cells were passaged at least two times before conducting an experiment. The use of freshly thawed cells was limited to 12 passages.

N9 microglial cells [426] were obtained from Dr. Fabio Bianco (Neuro-Zone, Italy) and were cultured in ISCOVE medium containing 10% FBS and 100 U/ml penicillin and streptomycin. Cells were passaged at a ratio of 1:6 every other day to obtain consistent results. The confluency of these cells was maintained at 50-70 % at all times. Freshly thawed cells were were used for a maximum of 15 passages.

HEK293T cells were purchased from ATCC and HT22 mouse hippocampal neuronal cells[58] were obtained from Dr. Shaohua Yang, (University of North Texas Health Sciences Center). Both were grown in DMEM containing 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin. All cell lines were grown at 37^{o}C in 5% $CO_{2.}$

2.2 **Plasmids and Lentiviral vectors**

The cDNA coding for mouse Syk-EGFP fusion protein was cloned into a puromycin resistance lentiviral vector pLVX as described (427). Lentivirus particles were generated by cotransfecting HEK293T cells (2 $X10^6$ cells/10 cm plate) with 5 µg of Syk-EGFP pLVX, 5µg of pHR' CMV-R8.20 VPR and 2.25 µg of pHR' CMV-VSVG using Lipofectamine 200 (Invitrogen). After 48 h the media supernatant containing viral particles was collected and filtered using 40 µm pore size filter. Harvested lentiviral particles were added to BV-2 cells (2 $X10^5$ cells/10 cm plate) in the presence of 10 µg/ml polybrene. 48 h post infection, cells were selected in the presence of 5 µg/ml puromycin for 92 h. Finally, cells were maintained in 2.5 µg/ml puromycin and passaged every 48 h.

To establish the mouse Syk knockdown BV-2 cell line, BV-2 cells (2 $X10^5$ cells/10 cm plate) were infected with one of a set of six independent GIPZ mouse Syk shRNA lentiviruses (GE Dharmacon). A scrambled shRNA was also infected to establish a control cell line. Six independent Syk shRNA and scrambled shRNA lentiviral particles were generated as described above with the lentiviral vectors for Syk-EGFP. Lipofectamine 3000 (Invitrogen) was used instead of Lipofectamine 2000 to transfect HEK293T cells. One of the plasmids among the six exhibited robust knockdown as evaluated by western blotting. Syk shRNA #5 plasmid was used to conduct the future experiments.

2.3 **Primary cell culture and animal models**

Primary microglial cells were isolated essentially as described (428, 429) from 1 month or 20 month old wild-type or *Syk*$^{+/-}$ C57BL/6 mice. Syk-haplosufficient mice were generated by gene targeting by Ingenious Targeting Laboratory, Ronkonkoma, NY. Approximately, 5 mice from each group were anesthetized using isoflurane anaesthetizing equipment and perfused in the left ventricle at constant pressure for 5 min with ice cold Ringer's solution containing 2 U/ml heparin (Cat. No. AK3004, Akron Biotech, USA). Immediately brains were aseptically removed and meninges separated. The brain was minced and dissociated with a sterile scalpel blade in media containing 1 mg/ml papain, 1.2 U/ml dispase II (Cat. No. P4762 and D4693, Sigma-Aldrich, USA) and 20 U/ml DNase1 (Cat. No AK3778, Akron Biotech, USA). Following pH neutralization of media, cells were pelleted and recovered, pipetted up and down using glass Pasteur pipettes of decreasing hole sizes, filtered through 70 µm and 40 µm cell strainers and collected in media. Cells were separated on a discontinuous percoll (Cat. No. 17-0891-01, GE Healthcare, USA) density gradient, collected, washed, and cultured on 1µg/cm^2 fibronectin (Cat. 341631, Calbiochem)-coated dishes. Three different concentrations (70% SIP, 37% SIP and 30% SIP) of saturated isotonic percoll (SIP) were used and microglia were present between 37% and 70% SIP. Isolated cells were washed and cultured in DMEM containing 5 ng/ml murine carrier free granulocyte and macrophage colony stimulating factor (GM-CSF) (Cat. No. 130-094-043, Miltenyl Biotech), 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin. For experiments, 1 X10^3 cells/well in a 24 well plate were grown in high glucose DMEM

containing 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin. Primary microglia were confirmed by western blotting and immunofluorescence staining of IBA1 (428). All mice were housed and experiments were done in accordance to the guidelines of IACUC at Purdue University.

2.4 **Stress granule formation**

BV-2 or N9 cells (1 $X10^4$ cells/well in a 24 well plate) were grown on poly-D-lysine coated cover slips in media containing 1% FBS for 12 h before treatment with sodium arsenite (SA) (1 μM), Aβ(1-42) (100 nM), or Aβ(1-42) fibrils (100 nM) for the indicated times. Aβ(1-42) (rPeptide, Cat. No. A-1008) or Aβ(42-1) (American Peptide Company, Cat. No. 62-0-81) were resuspended in DMSO, 1% NH_4OH at a concentration of 0.025 M. To prepare Aβ fibrils, DMSO soluble Aβ was sonicated for 30 min at 1 min intervals, shaken at 37^{o}C for 48 h and then at room temperature for 72 h (59, 430). Treated cells were fixed in 4% formaldehyde in PBS, permeabilized in 1% Triton X-100, blocked with 5% goat or donkey serum (depending on the species of origin of the secondary antibody) and incubated overnight at 4^{o}C with primary antibody diluted at a ratio of 1:1000 in 5% goat or donkey serum in PBS. Secondary antibodies Alexa Fluor 488 or 594 against mouse, rabbit, goat and donkey species were purchased from Life Technologies. Nuclei were visualized by staining with Hoechst 33342 (Sigma-Aldrich). Coverslips were mounted with ProLong® Gold Antifade reagent (Life Technologies) and examined using a Zeiss LSM 710 confocal microscope. Five 25X frames (average of 75 cells each frame) were randomly selected from three independent experiments and regions surrounding

SGs were selected, and corrected total cell fluorescence (CTCF) was calculated using ImageJ. CTCF was used to measure the SG area in arbitrary units also known as brightness values. The number of SGs was manually calculated from five random 63X frames (average of 25 cells each frame) from three independent experiments using Zeiss 2012 software. All statistical calculations were done using GraphPad Prism 6 and one way ANOVA statistical test was performed to calculate the *P*-value. Error bars in the figures represent means ± SEM.

2.5 **Western blotting and immunoprecipitation assays**

For general immunoblotting experiments, N9, BV-2 or primary microglial cells were lysed in buffer containing 1% NP-40, 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5X protease inhibitor cocktail (13911, Sigma-Aldrich), 10 µM sodium orthovandate for 15 min on ice. After centrifugation at 14000 X *g* for 10 min, supernatants were collected, separated by SDS-PAGE and analyzed by western blotting. To prepare soluble and insoluble fractions, cells were lysed in buffer A (15 mM Tris-HCl, pH 7.6, 0.3 M NaCl, 15 mM $MgCl_2$, 1% Triton X-100, 10 mM Ribonucleoside Vanadyl Complex (Cat. No. S1420S, New England Biolabs), 5X protease inhibitor cocktail and 10 µM sodium orthovanadate) on ice for 10 min. Cells were disrupted by mortar and pestle (20 times moved up and down). The insoluble fraction was isolated by centrifugation at 1500 X *g* for 7 min and the supernatant was collected as the soluble fraction. The insoluble fraction was dissolved in SDS-sample buffer (1% SDS in 1X NP-40 lysis buffer). Further shearing was obtained by passing the cell lysate through a syringe 10 times (28G needle).

Finally western blotting was performed to quantify the protein levels. For phosphotyrosine immunoprecipitation assays, whole cell lysates prepared in buffer A were incubated with anti-phosphotyrosine (4G10, EMD Millipore)-coated protein G magnetic beads (Cat. No. P3296, Sigma-Aldrich) for 2 h at 4^{o}C. Beads were washed thrice with 1X NP-40 lysis buffer, once with 1X high salt solution and once with double-distilled water. Finally, bound proteins were eluted with SDS-sample buffer. Immune complexes were examined by western blotting to identify associated proteins.

GFP-TRAP immunoprecipitation assays were conducted to isolate the GFP-fusion protein from Syk-EGFP-expressing BV-2 cells. Whole cell lysates were prepared in buffer A. A nucleus-enriched portion was separated by centrifuging at 5000 X *g* for 7 min at 4^{o}C. Supernatant was collected and incubated with GFP-TRAP beads (Cat. No. gta-20, ChromoTek Inc. USA) for 30 min at 4^{o}C. Beads were washed as described above and subjected to western blotting for further analysis.

2.6 **Microglial cell functional assays**

Phagocytic activity of N9 and BV-2 cells (1 X10^{4} cells/ plate) was assessed by the uptake of pHrodo^{TM} Red E. coli BioParticles® (Cat. No.P35361, Life Technologies). N9 and BV-2 cells were grown in poly-D-lysine coated glass bottom dishes (MatTek, P35G-1.5-10-C, MatTek Corporation, USA) for 12 h in growth media containing 1% FBS and then treated as indicated for 120 h. Subsequently media was removed. Cells were washed twice with 1X PBS buffer and incubated in 2 ml Live Cell Imaging Solution (Cat. no. A14291DJ, Life Technologies) containing 100 µl pHrodo^{TM} particles (431). Live imaging

was obtained thereafter in 5% CO_2 and 37^0C on a confocal microscope (Zeiss LSM 710) stage for another hour. Hoechst dye was added to visualize the nucleus. Live images of cells were taken at 30 sec intervals and compiled into a movie. For fixed cell images, cells were incubated for 1 h in 2 ml Live Cell Imaging Solution containing 100 µl pHrodo™ particles; fixed and examined by confocal microscopy. Phagocytosis of fluorescent red particles was quantified by measuring the mean corrected fluorescence intensity using ImageJ software from five random equal sized frames for each treatment condition.

The phagocytosis of FITC-labeled Aβ fibrils (rPeptide, Cat. No. A-1119-1) was measured using N9 cells in a similar manner except cells were incubated for 1 h with 25 µl FITC-labeled Aβ fibrils (fibrils prepared from 0.25 M solution of soluble Aβ(1-42)). Cells were washed extensively, fixed and imaged by confocal microscopy as mentioned above.

Intracellular ROS production was measured using both the 5(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) (Cat. No. 6982, Setareh Biotech) and dihydrorhodamine 123 (D123) (Cat. No. D-23806, Life Technologies) reagents. N9 cells were grown in 96-well plates in media containing 1% FBS and treated as indicated with SA or Aβ(1-42) in the presence or absence of 500 nM Syk kinase inhibitor (R406 or PRT-060318 (Selleckchem)) for 120 h. Carboxy-H2DCFDA (10 µM) was added and incubated at 37^oC for 1 h. Cells were washed 3 times with 1X PBS. Fluorescence was measured using a microplate reader (Biotek Synergy 4). Alternatively, treated and control cells were incubated with D123 (5 µM) for 1 h. Cells were stained with Hoechst dye, fixed and examined by confocal microscopy (Zeiss LSM 710)(91, 432).

Quantitative analyses used ImageJ to measure mean fluorescence intensity. The area enclosing the green fluorescent signal (background signal subtracted) was measured in 5 random frames from three independent experiments.

Extracellular production of H_2O_2 was measured using Acridan Lumigen PS-3 reagents (Amersham ECL kit) as described (433). ALPS-3 substrate was prepared by mixing Reagent A (H_2O_2, Amersham ECL kit) with Reagent B (Acridan solution, Amersham ECL kit) in a ratio of 10:1. N9 cells grown in media containing 1% FBS were plated on 1% poly-D-lysine coated 24 well plates and treated with SA, Aβ (1-42) and/or Syk inhibitors for 120 h. 50 µl of media from each well was transferred to another 96 well plate and 50 µl of fresh ALPS-3 substrate was added. The plate was stored in the dark for 10 min at room temperature. Chemiluminescence was detected using a luminescence plate reader (Glomax, Promega) at 430 nm.

To measure reactive nitrogen species, N9 cells were grown and treated as mentioned above. Media was collected and reactive nitrogen species quantified by measuring nitrite levels using the Measure-iT™ High-Sensitivity Nitrite Assay Kit (Cat. No. M36051, Life technologies). In a black opaque clear bottom 96 well microplate (Corning, USA), 100 µl of quantitation reagent was incubated with 10 µl of media from each well containing cells (post-treatment). After incubation for 10 min, 5 µl of the developing reagent was added and fluorescence was measured using a microplate reader (excitation/emission 365/450 nm, (Biotek Synergy 4)). A standard curve was plotted using the standards provided with the kit.

2.7 **Microglia-neuron co-culture and Annexin V assay**

N9 microglial cells (5 $X10^3$ cells/well) plated in poly-D-lysine-coated 24-well plates were treated as indicated with SA, Aβ and/or Syk inhibitor for 120 h. Cells were gently detached and washed with PBS. 5 $X10^2$ cells from each treatment condition were added to an 8 µm pore size transwell insert (Thincert^{TM,} Greiner bio-one) and placed atop 1.5 X 10^4 HT22 cells plated in DMEM media without phenol red containing 1% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin. Co-cultured cells were incubated for 48 h. After 48 h, HT22 cells were washed with PBS and incubated with FITC Annexin V (Cat. No. 556420, BD Biosciences) for 30 min at 37^{o}C. Finally cells were fixed, stained with Hoechst dye and examined by confocal microscopy.

2.8 **Human AD clinical samples**

Paraffin-fixed human AD brain and normal brain cortex samples were obtained from the Brain and Body Donation Program, Banner Sun Health Research Institute, Arizona[64]. Samples were clinically evaluated based on several criteria including mini-mental state examination (MMSE) score, plaque density, lewy body prevalence and dementia. Paraffin fixed cortex brain slices were heated at 65^{0}C for 30 min, fixed with 4% paraformaldehyde, washed sequentially with different concentrations of ethanol (70%, 50%, 30%) and then subjected to antigen retrieval by heating in 10 mM Tris/HCl (pH 10) for 20 min at 85^{0}C. Slides were blocked with 5% donkey serum and 2% BSA overnight at 4^{0}C. Brain slices were stained using primary antibodies against SYK, IBA1, G3BP and phosphotyrosine overnight at 4^{o}C. On the following day, slides were washed 5 times with PBST, stained with Alexa Fluor secondary antibodies, fixed with VectorMount AQ (Cat.

H-5501, Vector Labs) and examined using a Zeiss 710 LSM confocal microscope. The area enclosed by SG in brain slides was measured using ImageJ as mentioned earlier for SGs in cultured cells.

2.9 **Antibodies**

The antibodies and sources used in this study were as follows: Syk (D3Z1E, Cell Signaling Technology or B01P, Abnova), G3BP1 (611126, BD Biosciences or 07-1801, EMD Millipore), pEIF2α (E90, Abcam), TIA-1(ab2712, Abcam), PABP1 (ab21060, Abcam), GFP (168AT1211, Abgent), GAPDH (6C5, Ambion), pTYR (4G10, EMD Millipore), IBA1 (ab5076, Abcam), GFAP (GF5, Abcam), TDP-43 (10782-2-AP, Proteintech), NOS2 (ab3523, Abcam), Integrin β1 (4B7R, Santa Cruz Biotech), CD32 (ab197930, Abcam), and Rabbit IgG (Sigma-Aldrich).

CHAPTER THREE: RESULTS

3.1. **SYK is highly expressed in BV-2 and N9 microglia cell**

A few studies have suggested that SYK is present in microglial cells, but none of these compared the expression levels of SYK to those of other immune cells. Endogenous SYK protein level had not been examined previously in N9 cells. Hence, I first wanted to confirm the expression level of SYK in two well- known immortalized microglial cell lines: BV-2 and N9. Cells were grown for 24 h in complete media and lysed in 1X NP-40 lysis buffer. Immunoblotting for endogenous SYK revealed a high level of expression of SYK in both lines as shown in comparison to DG75 B cells (Fig. 3.1). It has been previously observed in our lab that DG175 B cells express substantial levels of SYK .

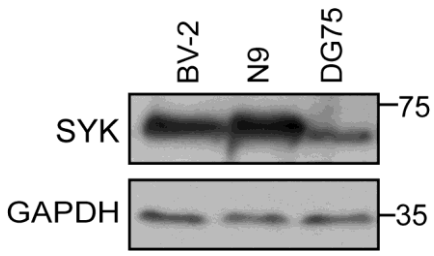

Figure 3.1 SYK is highly expressed in N9 and BV-2 microglial cell

Figure 3.1 SYK expression was examined in the DG75, B cell-line, and the BV-2 and N9 microglial cell lines. Cell lysates were analyzed by western blotting with SYK and GAPDH antibodies. GAPDH was detected as a loading control.

3.2 **Low dose of sodium arsenite promotes SG formation in microglial cells**

Environmental stress induces SG formation in eukaryotic cells. One of the classical SG inducers is sodium arsenite (SA). To determine if SGs form in these MG, I first treated BV-2 and N9 cells with sodium arsenite for 24 h. Numerous SGs were found in both cell lines when treated with low concentrations of SA (1 μM) for 24 h as revealed by the appearance of puncta that stained with the SG marker G3BP1 (Fig. 3.2). Next I wanted to examine if SYK localized to these SGs. To determine that, I treated both MG as described above in Fig.3.2, and fixed and stained cells with an antibody against the kinase. A fraction of SYK co-localized with G3BP1 in SGs in both cell types when treated with SA (Fig. 3.2). The concentration of SA that was required to induce SGs was surprisingly low compared to the level commonly (250/500 μM) used to induce SGs in other cell types (417, 434, 435).

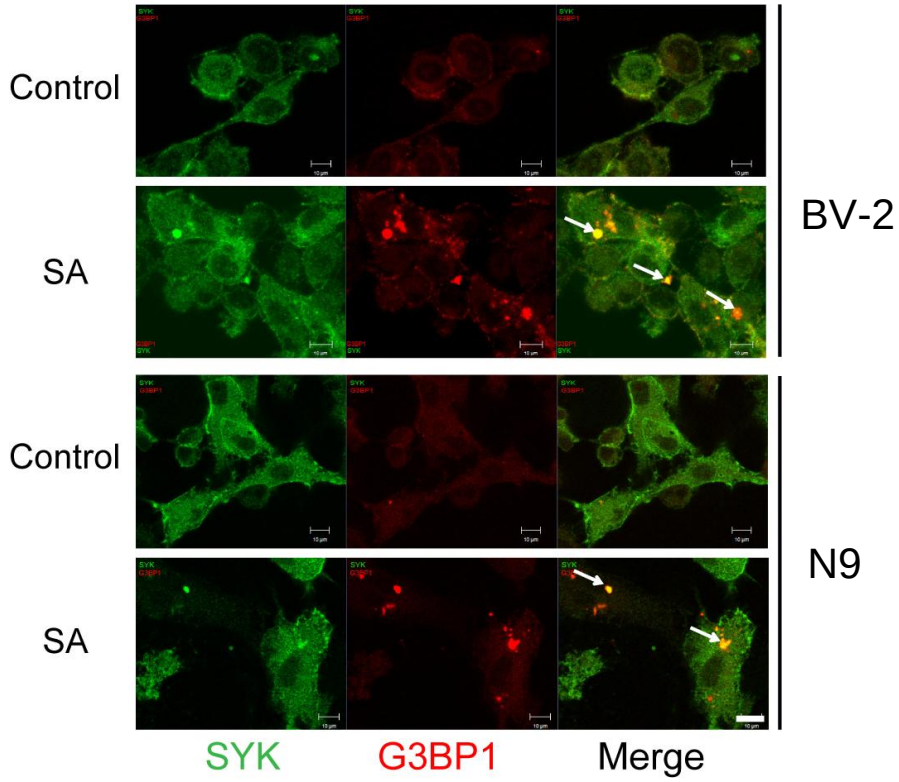

Figure 3.2 SA stimulation manifests SG formation in N9 and BV-2 cells

Figure 3.2 SG formation in BV-2 and N9 cells was examined under SA treatment. BV-2 and N9 cells were treated with SA (1 μM) for 24 h. On the following day, cells were fixed and stained for endogenous SYK (green) and G3BP1 (red). Confocal images were obtained using a Zeiss LSM 710 microscope. White arrows indicate SYK and G3BP1 co-localized SGs. Scale = 10 μm.

3.3 **Soluble amyloid beta induces SG formation in MG**

Recent reports have shown that misfolded or aggregated proteins like TAU, TDP-43 and PrP can induce SG formation in neuronal cells (38, 436, 437). Neurodegenerative disease animal models have also confirmed the presence of misfolded proteins associated in SGs in neuronal cells. It is hypothesized that stressors in neurogeneration can induce such SGs. I then asked if exposure to a stressor implicated in neurodegeneration might also lead to SG formation in MG. Abundant SYK-positive SGs were induced in both BV-2 and N9 cells treated with 100 nM soluble Aβ(1-42) for 24 h (Fig. 3.3A). Quantitative analyses of SYK and G3BP1 co-localized SG punctas were made for BV- 2 cells using ImageJ. Analyses revealed a five-fold increase in area containing SYK and G3BP1 positive SG in cells treated with SA or Aβ(1-42) compared to control (Fig. 3.3B). As a control, BV-2 cells also were treated with soluble Aβ(42-1). Aβ(42-1) treatment did not form SGs in BV-2 cells (Fig. 3.3C)

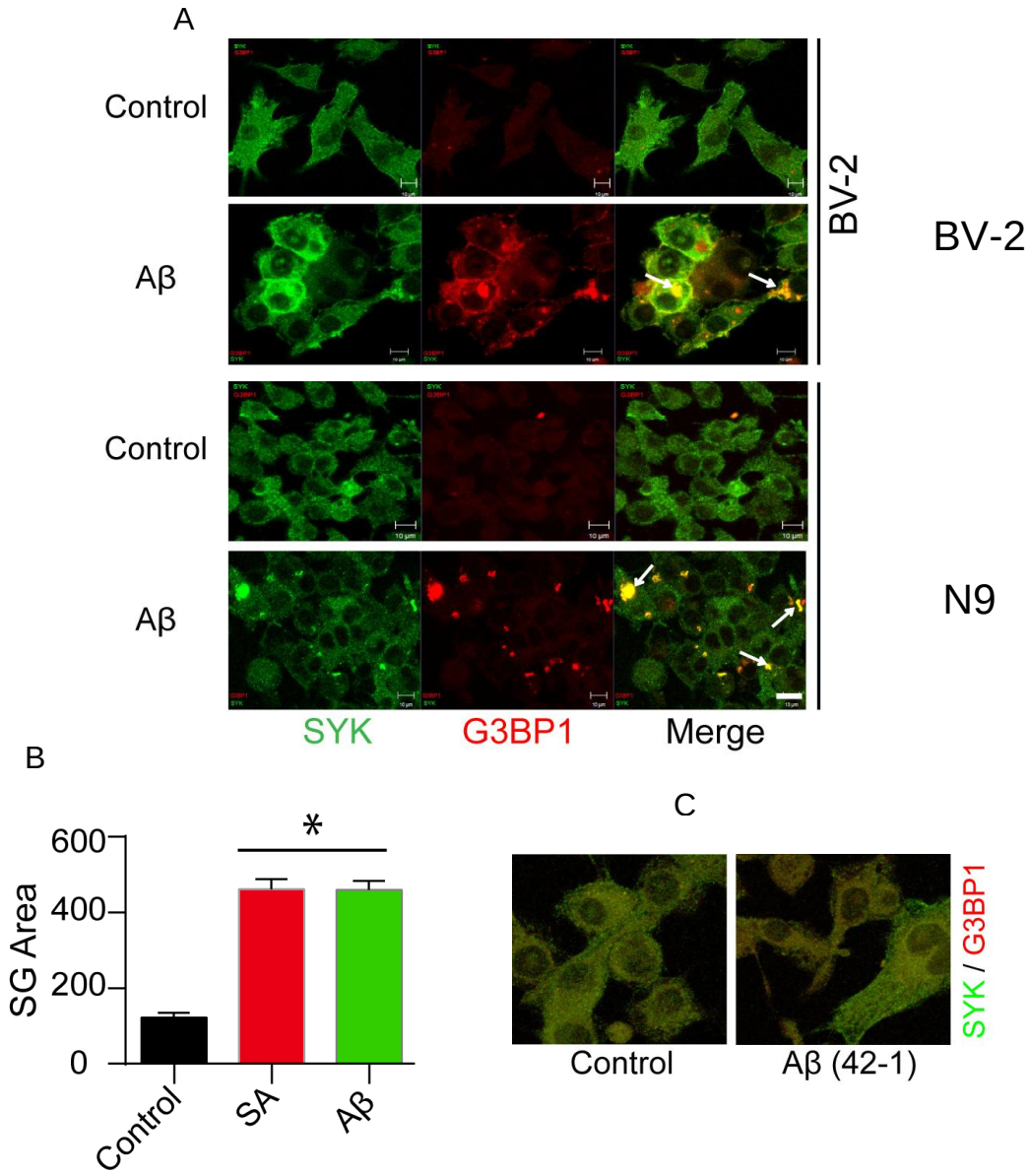

Figure 3.3 Aβ stimulation manifests SG formation in N9 and BV-2 cells

Figure 3.3. (A) BV-2 and N9 cells were treated without (Control) or with Aβ for 24 h, fixed and stained for SYK (green) and G3BP1 (red). Examples of SGs are indicated by the arrows. (B) The area occupied by SYK and G3BP1 positive SGs in N9 cells was quantified by Image J analysis of 5 random frames from 3 independent experiments. Results represent means ± SEM. *P = 0.001. (C) BV-2 cells were treated with soluble A□(42-1) as a control for 24 h, fixed and stained for SYK (green) and G3BP1 (red). Only merged images are shown. Bar = 10 μm.

3.4 **SYK and G3BP1 co-localizes with other SG core components in BV-2 and N9 cells**

SGs are extremely dynamic and varied in terms of their components. Various translation initiating factors, mRNA-binding proteins and some other proteins that associate with SG core proteins form the major components of SGs. Depending on the biological system, cell line and stress type, different SG components have been reported. I then asked if other proteins commonly found in SGs were present within these complexes in MG. BV2 cells were treated with SA or Aβ for 24 h, fixed and stained for SYK and co-stained for phosphorylated EIF2α, PABP1, TDP-43 or TIA-1. SYK-positive puncta produced by both stimuli contained each of these proteins (Fig. 3.4A). Similar results were observed in N9 cells under similar treatment conditions (Fig. 3.4B). G3BP1 also co-localized in puncta with pEIF2α, TDP-43 and TIA-1 in N9 cells treated with SA or Aβ (Fig. 3.5).

Since SGs are insoluble in mild detergents, I fractionated control and stressed MG into detergent-soluble and insoluble fractions and immunoblotted each for SYK, G3BP1, pEIF2α and TIA-1. A significant increase in the presence of SYK, G3BP1, pEIF2α and TIA-1 in the detergent-insoluble fractions accompanied treatment of both N9 and BV-2 cells with either SA or Aβ (Fig. 3.6).

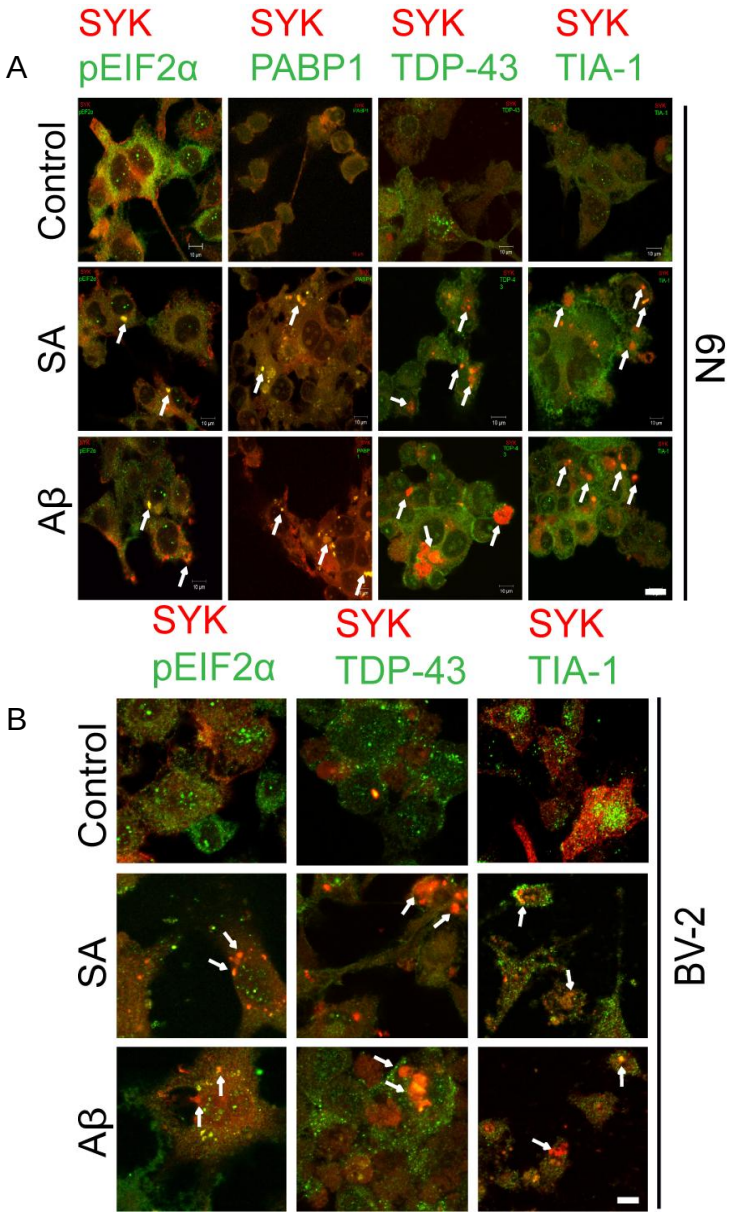

Figure 3.4 SYK co-localizes with pEIF2α, TDP-43, PABP1 and TIA-1 in BV-2 and N9 cells

Figure 3.4. BV-2 (A) and N9 (B) cells were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for SYK, pEIF2α, PABP1, TDP-43, TIA-1 and/or G3BP1 as indicated. Only merged images are displayed. Examples of SGs are indicated by the arrows. Bar = 10 μm.

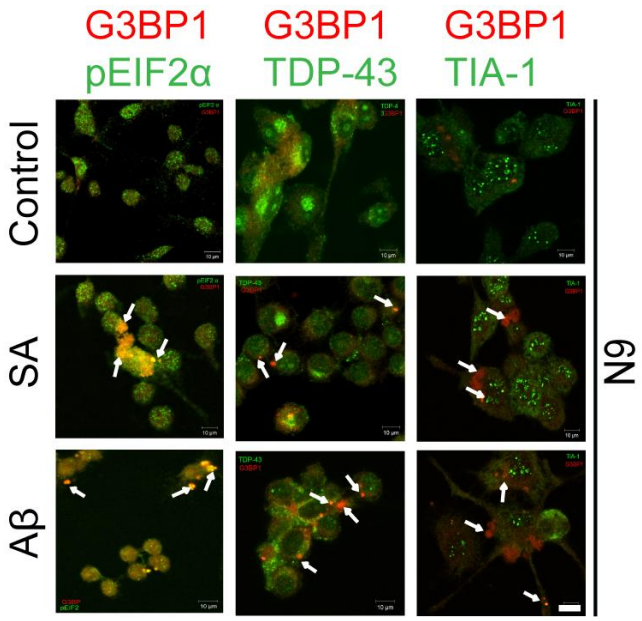

Figure 3.5. G3BP1 co-localizes with pEIF2α, TDP-43, PABP1 and TIA-1 in BV-2 and N9 cells

Figure 3.5. N9 cells were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for G3BP1, pEIF2α, TDP-43 and TIA-1. Only merged images are displayed. Examples of SGs are indicated by the arrows. Bar = 10 μm.

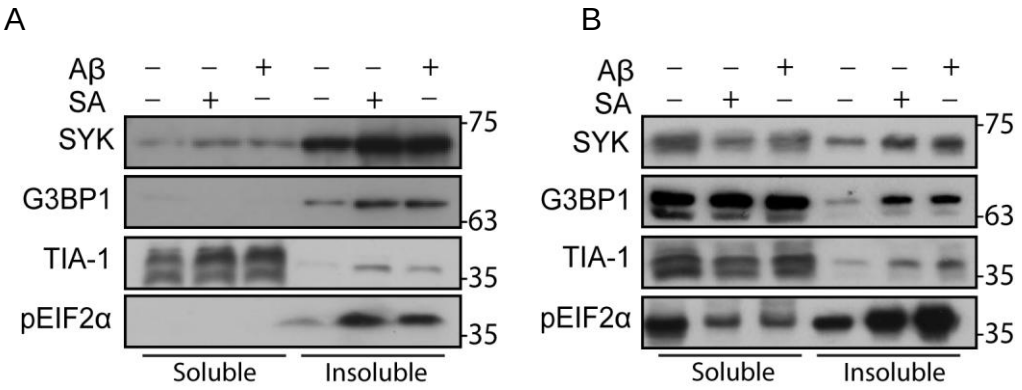

Figure 3.6 SG markers like SYK, G3BP1, TIA-1 and pEIF2α expression increases in SDS soluble fraction in BV-2 and N9 cells

Figure 3.6 N9 (A) and BV-2 (B) cells were treated without (-) or with (+) SA or soluble Aβ for 24 h and then separated into detergent soluble and insoluble fractions, which were analyzed by western blotting for SYK, G3BP1, TIA-1 and pEIF2α. SA and Aβ showed an increase in protein levels in insoluble fractions under treatment.

3.5 **SYK associates with other SG core components in BV-2 cells**

SG core components associate with other translation initiating factors, mRNA-binding proteins and proteins that interact with core components. To further confirm an interaction or association of SYK with SG markers, I expressed SYK-EGFP in BV-2 cells, which were then treated with SA or Aβ for 24 hours. Following treatment, cells were lysed and SYK-EGFP was immunoprecipitated using GFP-TRAP beads. Immunoprecipitated SYK-EGFP was immunoblotted for endogenous SYK, G3BP1 and pEIF2α. Both stress stimuli resulted in an increased association of SYK-EGFP with endogenous SYK, G3BP1 and pEIF2α (Fig. 3.7).

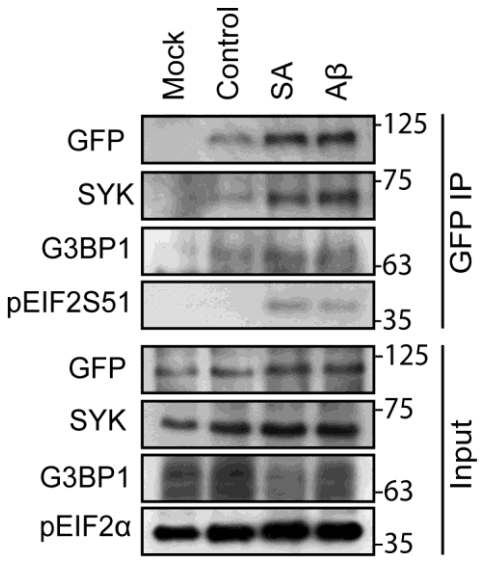

Figure 3.7 SG markers G3BP1and pEIF2α associate with GFP-tagged SYK in SYK-EGFP-expressing BV-2 cells

Figure 3.7 SYK-EGFP was immunoprecipitated from lentiviral-infected BV-2 cells using GFP-trap beads. Cell lysates (Input) and immune complexes were analyzed by western blotting using antibodies against GFP, SYK, G3BP1 and pEIF2α. Unmodified beads were used as a control (Mock).

3.6 **SYK is active when present in SGs**

SYK is a protein tyrosine kinase that phosphorylates its substrates to regulate signaling pathways. In immune cells like macrophages and microglia, active SYK mediates downstream signaling that could activate pathways producing reactive oxygen and nitrogen species. To determine if SYK was active when recruited to SGs, I stained control and SA- and Aβ-treated N9 and BV-2 cells with antibodies against SYK and phosphotyrosine. SYK-positive puncta induced by either stimulus contained abundant phosphotyrosine indicating that SYK was active in SGs (Fig. 3.8A and 3.9). Quantification of images confirmed a significant increase in area containing co-localized SYK and phosphotyrosine in treated compared to control N9 cells (Fig. 3.8B). Western blotting of lysates of control and SA- or Aβ-treated N9 cells with antibodies specific for the phosphorylated activation loop of SYK confirmed that SYK was active and phosphorylated in response to both stimuli (Fig. 3.8C). To further confirm the presence of active SYK in SGs biochemically, I immunoprecipitated tyrosine-phosphorylated proteins from control, SA- and Aβ-treated N9 cells using magnetic beads and immunoblotted the immune complexes for phosphotyrosine and SYK. Induction of SGs with either stimulus resulted in a significant increase in the phosphorylation of multiple proteins including SYK itself (Fig. 3.8D).

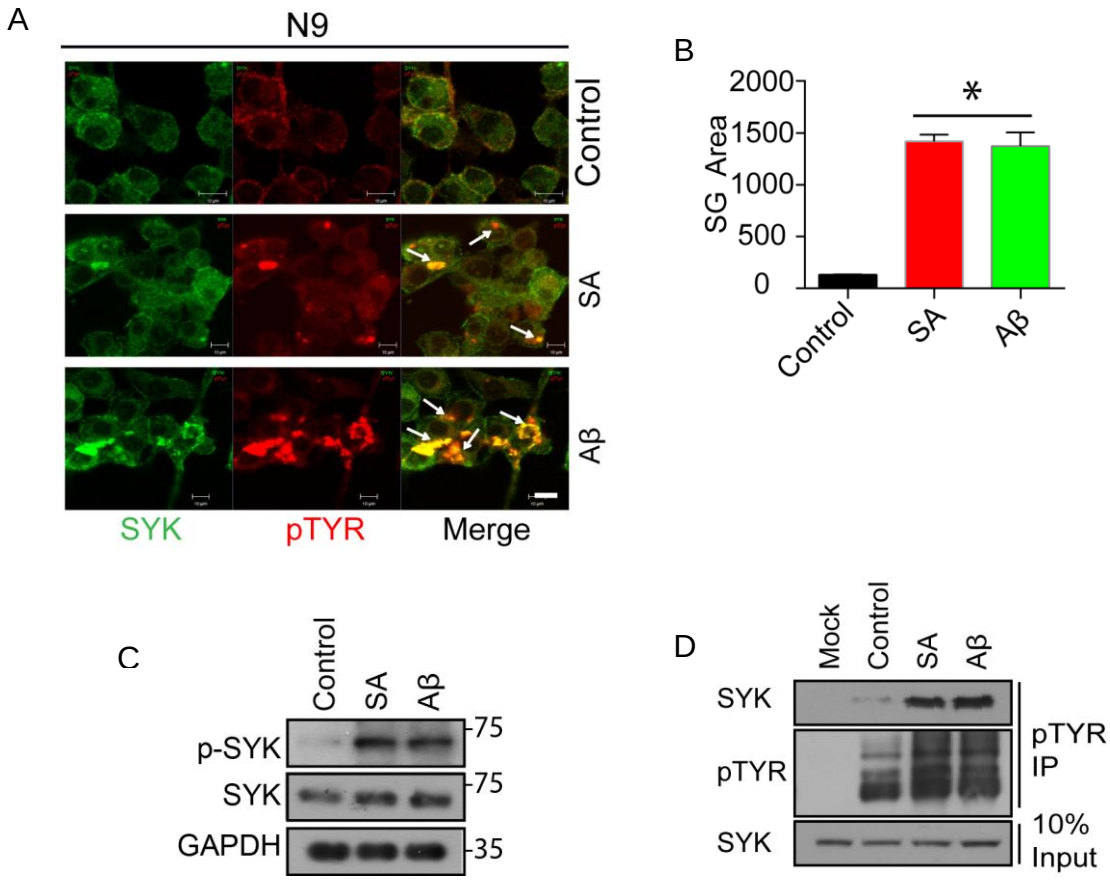

Figure 3.8 SYK is active when co-localized in SGs in N9 cells under treatment of SA and Aβ

Figure 3.8. (A) N9 cells were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for SYK (green) and phosphotyrosine (pTYR, red). Examples of SGs are indicated by the arrows. Bar = 10 µm. (B) The area occupied by SYK and phosphotyrosine positive SGs in (A) was quantified by ImageJ analysis of 5 random frames from 3 independent experiments. Results represent means ± SEM. *P = 0.001. (C) Lysates from N9 cells treated without (Control) or with SA or soluble Aβ for 24 h were analyzed by western blotting using antibodies against SYK phosphorylated on Y519 and Y520 (p-SYK), SYK or GAPDH.

(D) Phosphotyrosine-containing proteins were immunoprecipitated from lysates of N9 cells treated without (Control) or with SA or soluble Aβ for 24 h. Immune complexes were examined by western blotting with antibodies against SYK or phosphotyrosine (pTYR).

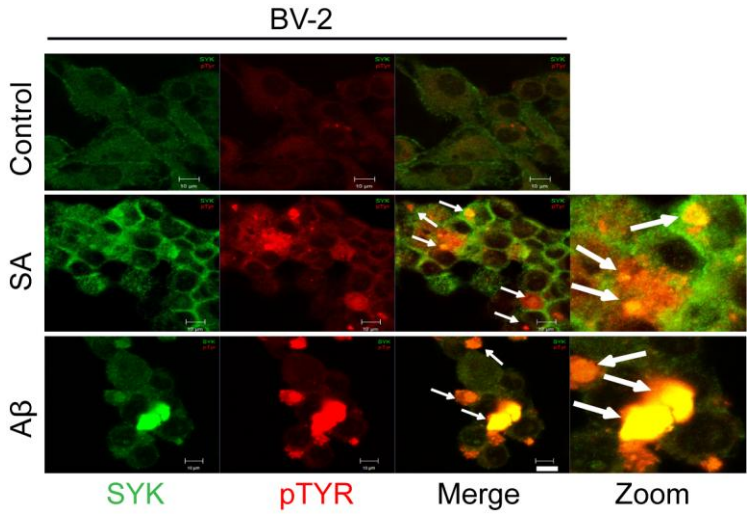

Figure 3.9. SYK is active when co-localized in SGs in BV-2 cells under SA and Aβ treatment

Figure 3.9 BV-2 cells were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for SYK (green) and phosphotyrosine (pTyr, red). Examples of SGs are indicated by the arrows. Bar = 10 μm.

3.7 **SYK regulates SG formation in MG under SA and Aβ treatment**

SG dynamics are a function of their formation and clearance. To determine if SYK plays an important role in SG formation, I stimulated BV-2 cells with SA or Aβ for 24 h in the absence or presence of a SYK inhibitor, either R406 or PRT318. In the presence of either inhibitor, neither SA nor Aβ induced the formation of significant SGs (Fig. 3.10A and 3.10B). The ability of each inhibitor to reduce SYK activity was confirmed by the western blotting with anti-SYK and antiphosphotyrosine antibodies of lysates of BV-2 cells treated with H_2O_2 in the presence or absence of R406 or PRT318. H_2O_2, an inhibitor of tyrosine phosphatases, led to the activation of SYK and its autophosphorylation on Y317 with a resulting shift in its electrophoretic mobility[37], which was lost in inhibitor-treated cells (Fig. 3.10C).

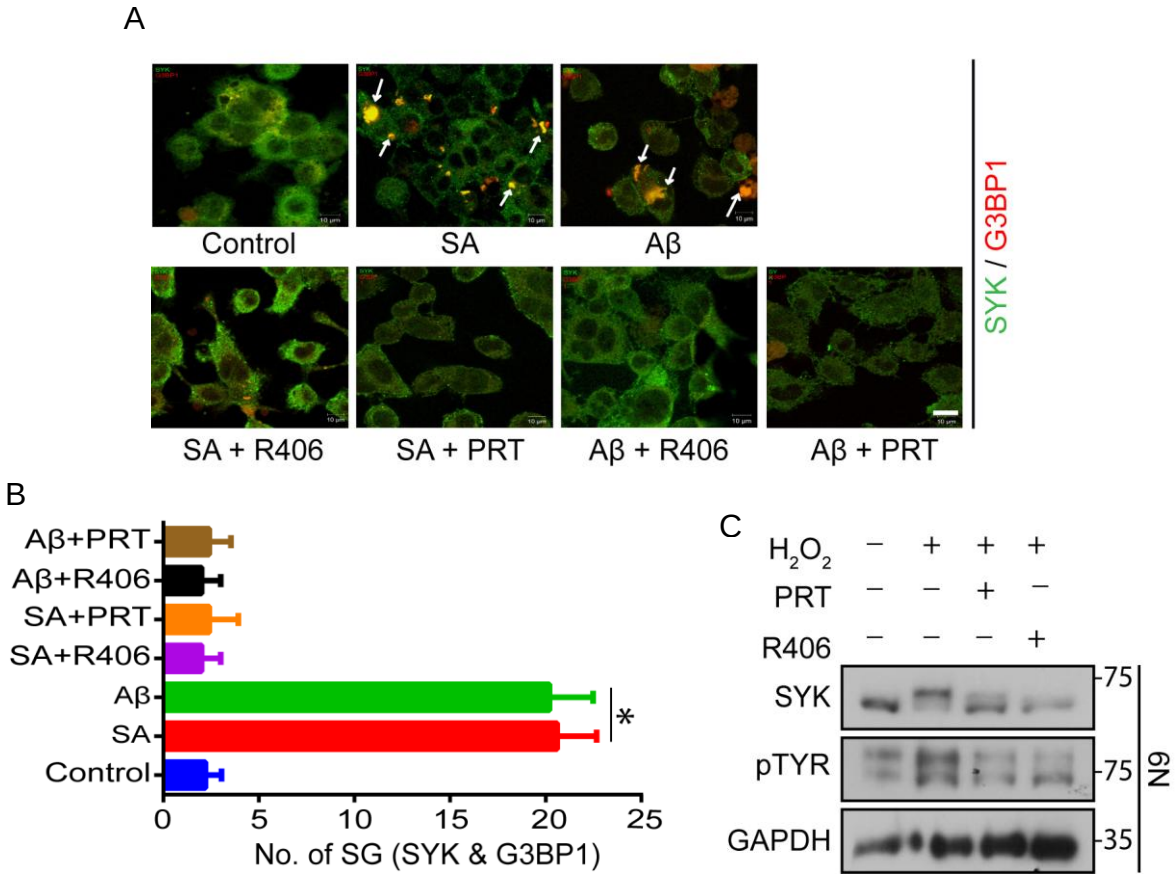

Figure 3.10 SG formation dynamics is affected by SYK

Figure 3.10. (A) N9 cells were treated without (Control) or with SA or soluble Aβ for 24 h in the presence or absence of R406 or PRT318 (PRT), fixed and stained for SYK (green) and G3BP1 (red). Examples of SGs are indicated by the arrows. Bar = 10 µm. (B) The average number of SGs formed in five random 25X frames were counted for each treatment. Results represent means ± SEM from 3 independent experiments. *P = 0.0001. (C) N9 cells were treated without (-) or with (+) H_2O_2 (5 µM) for 4 h in the absence (-) or presence (+) of PRT318 or R406 (500 nM each). Lysates were examined by western blotting for SYK, phosphotyrosine (pTYR) and GAPDH.

3.8 **Knockdown of SYK prevents SG formation in MG under SA and Aβ treatment**

SYK inhibitors reduced SG formation in N9 cells induced by SA and Aβ treatment. To confirm a direct role for SYK in SG formation, I infected BV-2 cells with lentiviruses expressing EGFP and either shRNA against SYK or a scrambled shRNA. SYK levels were selectively reduced in cells expressing the SYK shRNA (Fig. 3.11A) as confirmed by western blotting. Infected cells were treated with SA or Aβ for 24 hours, fixed and stained for G3BP1. While cells infected with scrambled shRNA readily formed SGs, cells with reduced levels of SYK failed to do so (Fig. 3.11B). Correspondingly, the appearance of SYK positive SGs was reduced in the knockdown cells (Fig. 3.12).

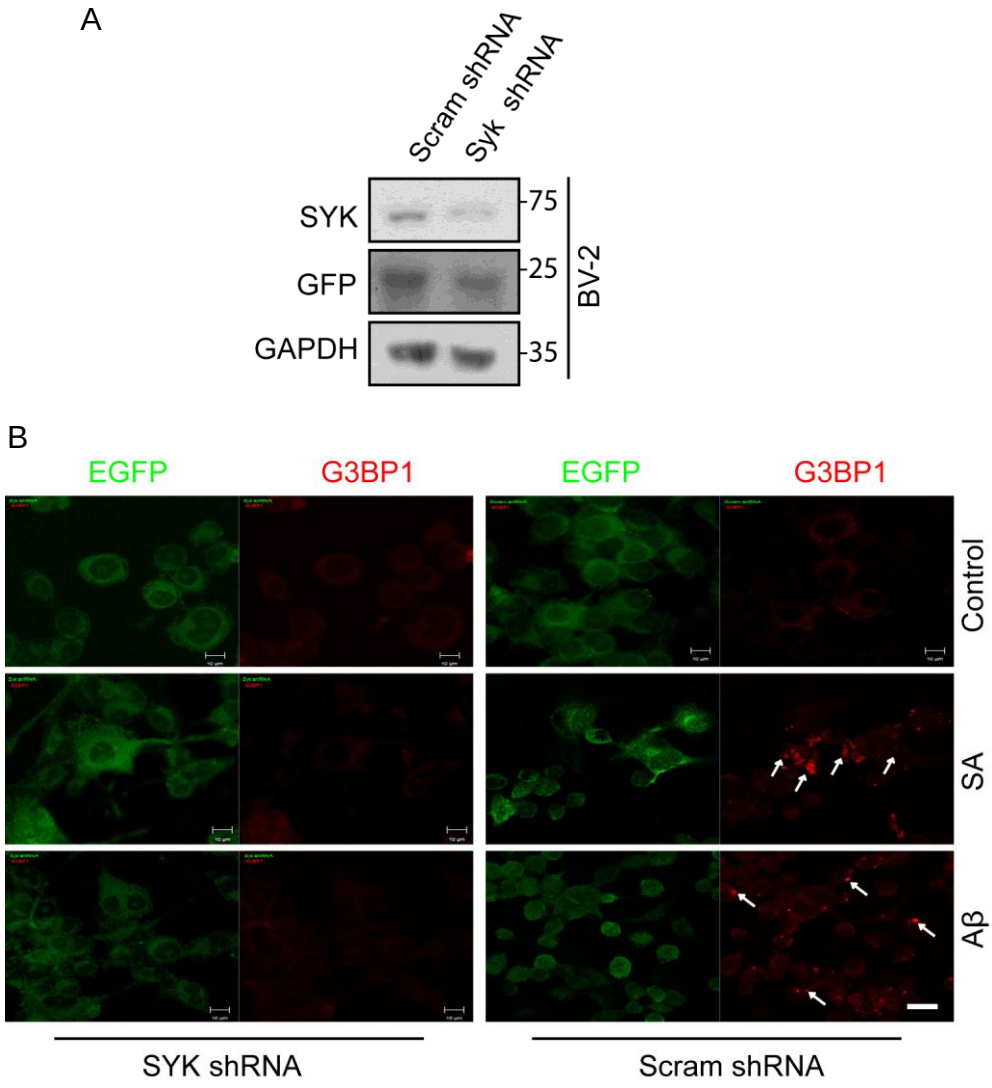

Figure 3.11 SYK knockdown prevents G3BP1 associated SG formation in BV-2 cells

Figure 3.11 (A) BV-2 cells expressing EGFP and scrambled shRNA or SYK shRNA were examined by western blotting with antibodies against G3BP1, GFP and GAPDH. (B) BV-2 cells infected with a lentivirus expressing EGFP and either a SYK or scrambled shRNA were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for G3BP1 (red). Examples of SGs are indicated by the arrows. Bar = 10 μm.

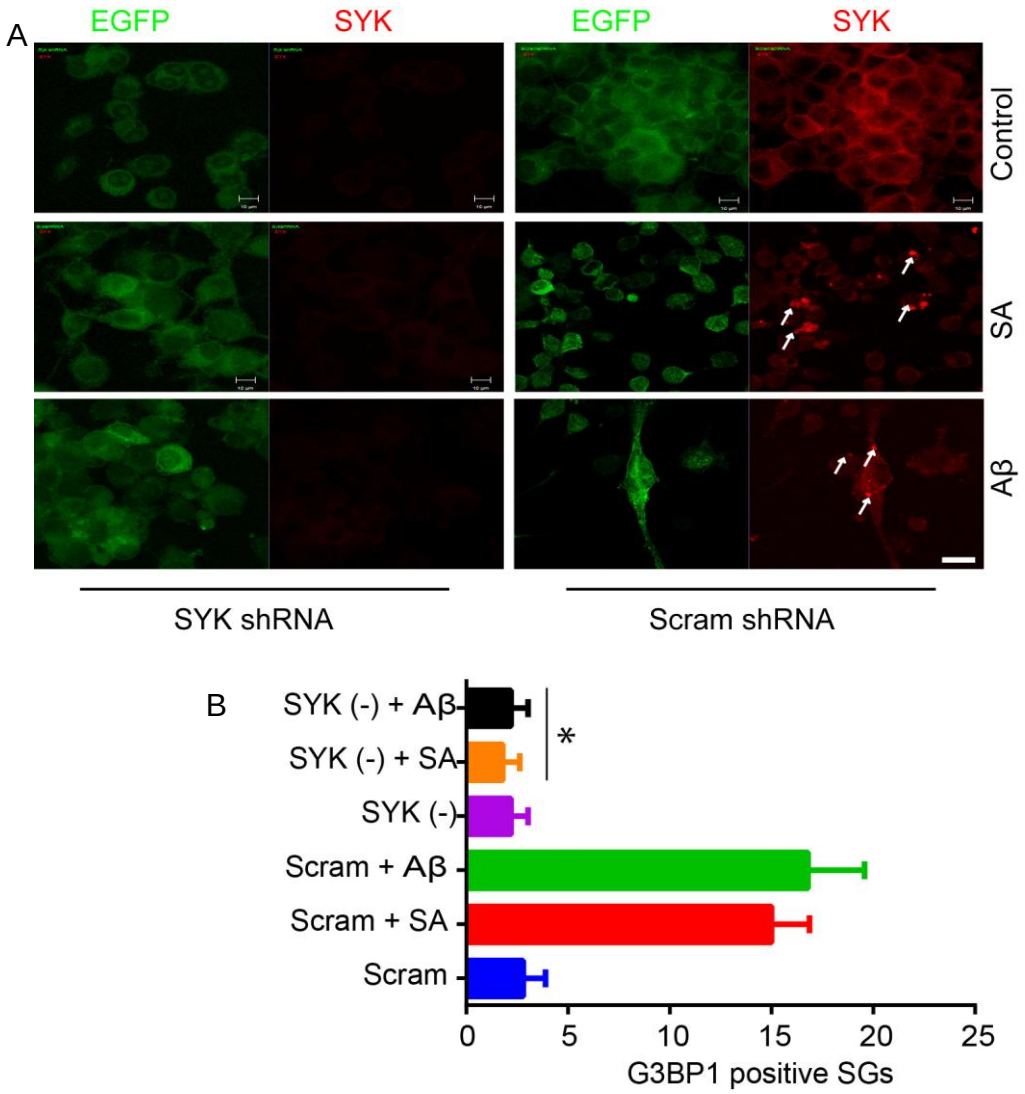

Figure 3.12 SYK knockdown prevents SYK associated SG formation in BV-2 cells

Figure 3.12 (A) BV-2 cells infected with a lentivirus expressing EGFP and either a SYK or scrambled shRNA were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for endogenous SYK (red). Examples of SGs are indicated by the arrows. Bar = 10 μm. (B) Quantification of G3BP1 positive SGs present within 10 random 25X frames for cells expressing SYK shRNA (SYK (-)) or scrambled shRNA (Scram). *P = 0.001.

MG cells lose their efficiency to phagocytose with age. Neurodegeneration is more prevalent in aged individuals. We used a haploinsufficient SYK mouse to conduct these studies. Since SYK knockout is lethal to pups, we had to use haplo-insufficient SYK mice to observe the role if microglial cells in old animal models. In order to first confirm that SYK was indeed knocked down in dead pups or reduced in haploinsufficient mice, we isolated liver cells from the mouse, lysed and immunoblotted for SYK and GAPDH (Fig. 3.13 B). To better understand the role of age on MG, I isolated primary MG from 20 month and 1 month old wild-type mice and haplosufficient mice. Isolated cells were positive for the MG marker IBA-1, but lacked the astrocyte marker GFAP (Fig. 3.13A and C). Cells were treated with SA or Aβ for 24 h, fixed and stained for IBA-1, SYK and G3BP1. Primary MG formed SYK- and G3BP1-positive puncta in response to both stress stimuli, which were particularly abundant in cells isolated from aged (20 month) animals (Fig. 3.14C). SG formation also was observed, albeit to a lesser extent, in cells isolated from 1 month old animals (Fig. 3.14A).

I also asked if the dependence of SG formation on SYK held true for primary cells. MG isolated from the brains of SYK haploinsufficient mice expressed half as much SYK as cells from wild-type mice (Fig. 3.13A). Since a complete SYK knockout was lethal as seen previously (296), I was unable to test cells from $Syk^{-/-}$ animals. SG formation was comparable in cells from one month old $Syk^{+/+}$ and $Syk^{+/-}$ animals (Fig. 3.14A, 3.15A and 3.16). However SG formation was reduced considerably in cells isolated from the brains of 20 month old $Syk^{+/-}$ mice as compared to their wild-type counterparts (Fig. 3.15C and 3.16).

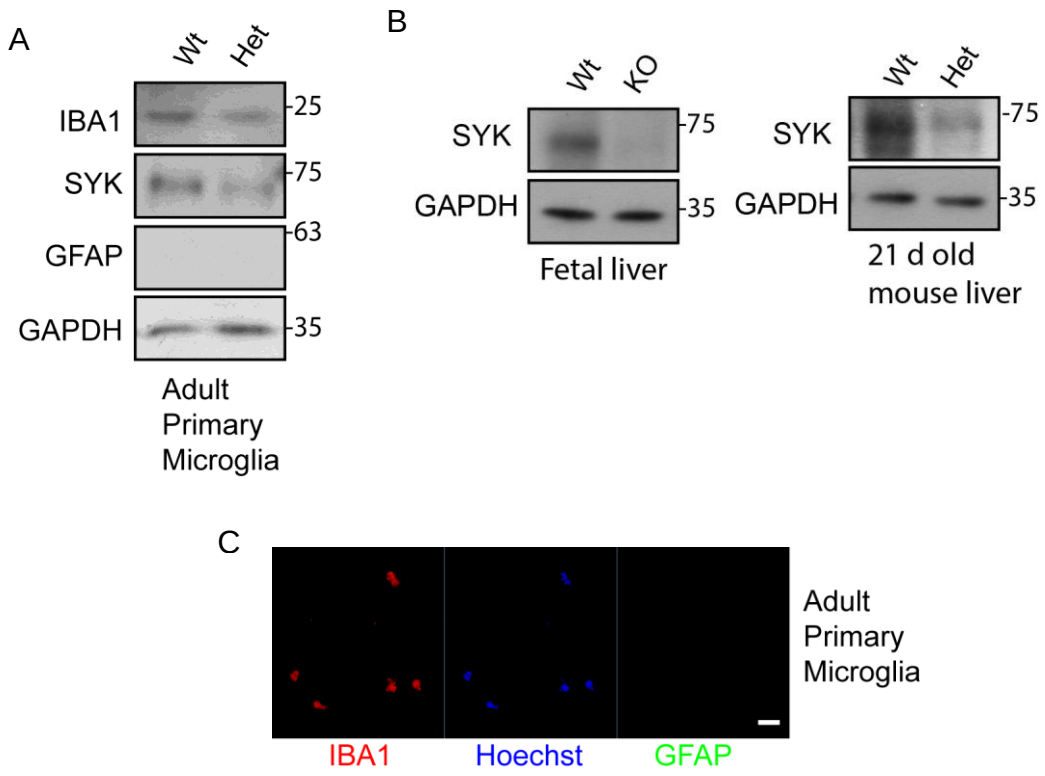

Figure 3.13 Primary microglial culture express IBA-1and haplosufficient mice express reduced SYK in them

Figure 3.13 (A) Adult primary MG were isolated from 1 month old wild-type (Wt) or *Syk*$^{+/-}$ (Het) mice and examined by western blotting with antibodies against IBA1, SYK, GFAP and GAPDH. (A) Lysates of cells from fetal livers isolated from wild-type (Wt) or *Syk*$^{-/-}$ (KO) mice or from livers of 21 day old wild-type (Wt) or *Syk*$^{+/-}$ mice were analyzed by western blotting for SYK and GAPDH. (B) MG were isolated from 1 month old wild-type (Wt) or *Syk*$^{+/-}$ (Het) mice and examined by immunofluorescence with antibodies against IBA1, Hoechst and GFAP. Bar = 10 μm.

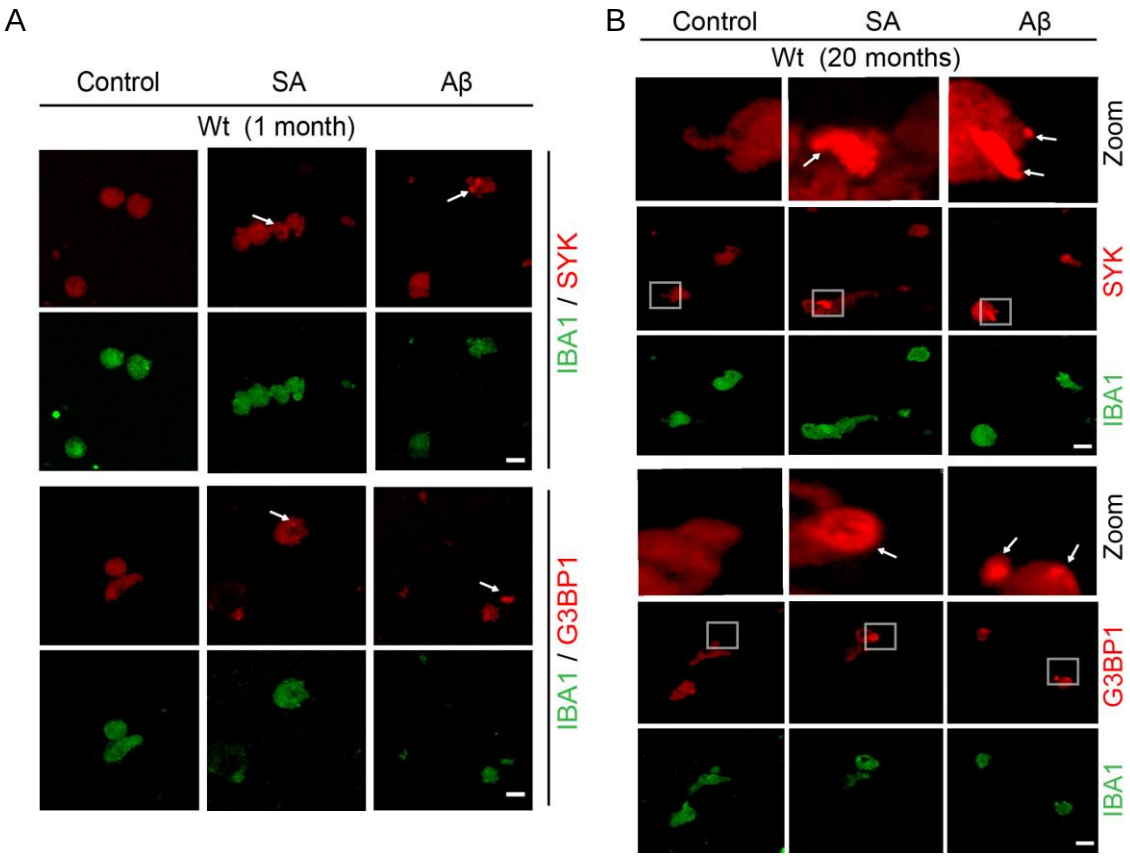

Figure 3.14 Adult primary MG from 20 month old mice generates large SGs

Figure 3.14. (A and B) MG isolated from the brains of 1 month old wild - type mice (A) or 20 month old wild-type mice (B) were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for SYK, IBA1 or G3BP1. Examples of SGs are indicated by the arrows. Bar = 5 μm.

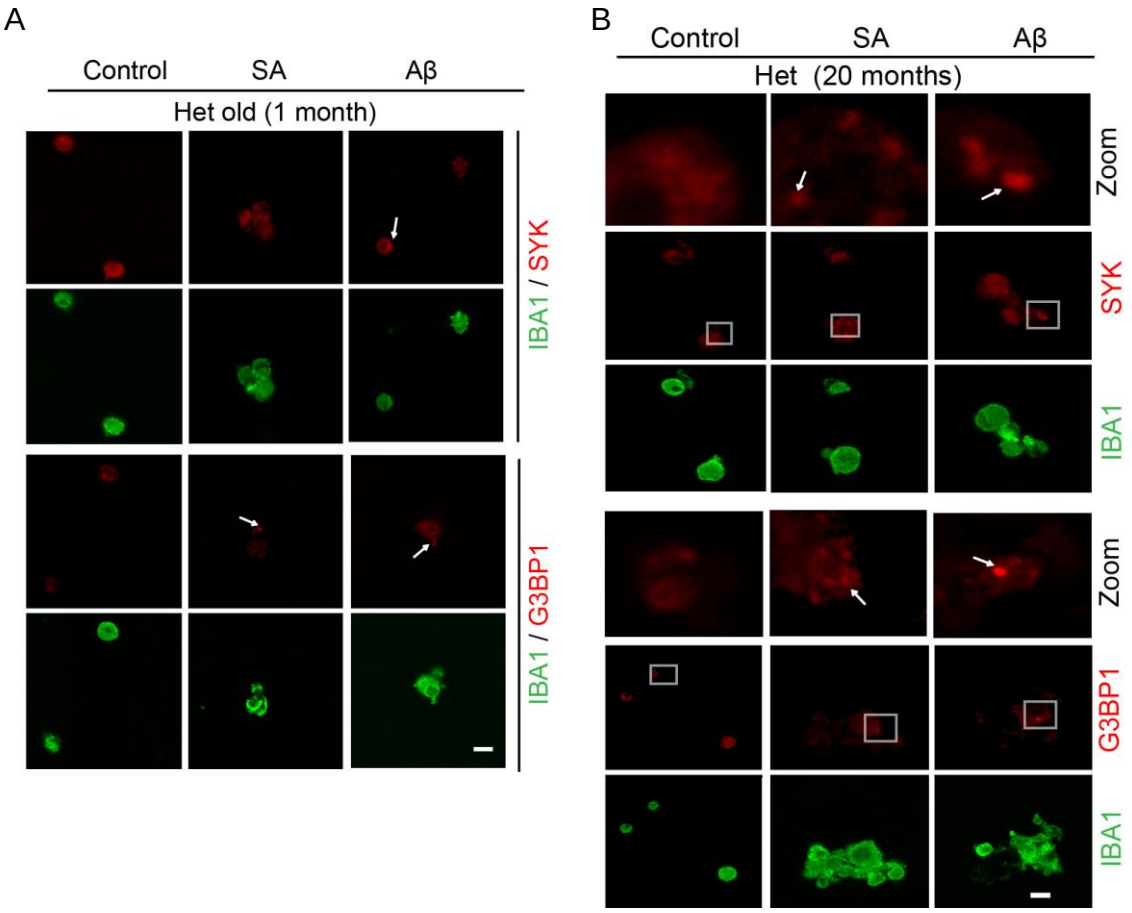

Figure 3.15 Adult primary MG from 20 month old mice Syk+/- generate large SGs upon stress induction compared to cells from 1 month old *Syk*$^{+/-}$ mice

Figure 3.15. (A and B) MG isolated from the brains of 1 month old *Syk*$^{+/-}$ mice (A) or 20 month old *Syk*$^{+/-}$ mice (B) were treated without (Control) or with SA or soluble Aβ for 24 h, fixed and stained for SYK, IBA1 or G3BP1. Examples of SGs are indicated by the arrows. Bar = 5 μm.

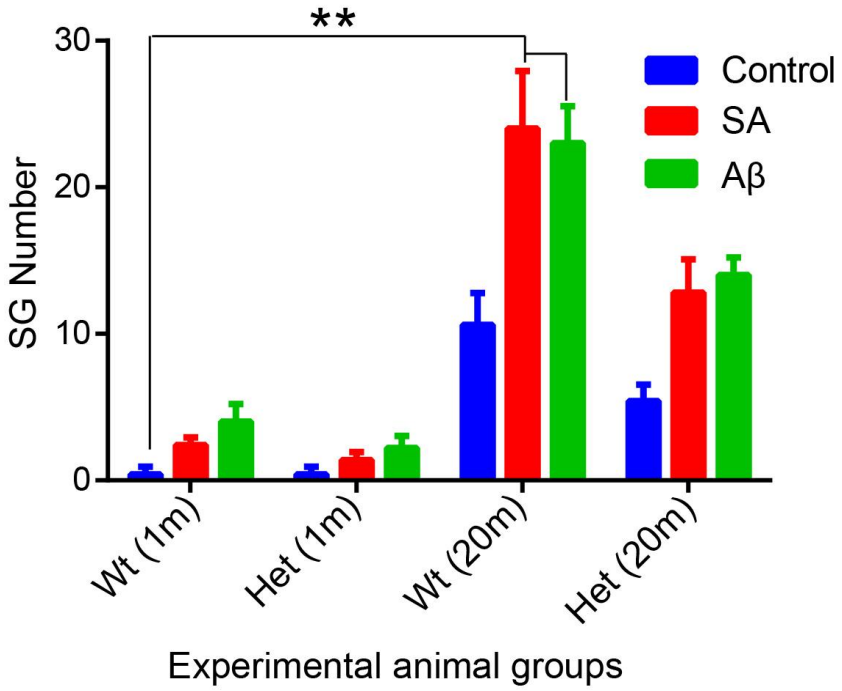

Figure 3.16 Quantitative comparison of SG formation in MG from young (1m) and old (20m), wild-type (Wt) and *Syk* haploinsufficient (Het) mice

Figure 3.16 The average number of SGs formed in five random 63X frames for SYK wild-type (1 month and 20 month) and $Syk^{+/-}$ (1 month and 20 month) (Fig. 3.13 & 3.14) for each treatment. Results represent means ± SEM from 3 independent experiments.

3.10 **Prolonged chronic stress induces large and irreversible SGs in N9 cells**

It has been proposed that exposure to chronic stress leads to the formation of persistent SGs in the brain (29). AD animal model P3106 brains have also shown increased SGs with severity of the disease. To examine this in MG, I exposed N9 cells to low levels of SA or Aβ for a period of five days to mimic chronic stress. Prolonged stress led to the formation of large SGs that contained both SYK and G3BP1 (Fig. 3.17A). To determine if the formation of these SGs was reversible, I removed the stress and allowed cells to recover for 48 h. While the intensity of staining for G3BP1 declined somewhat, the area within the cell occupied by SGs was largely unchanged (Fig. 3.17A and 3.17C). In contrast, most SGs formed in cells exposed to SA or Aβ for only 24 h were cleared within 24 h after removal of the stress stimulus (Fig. 3.17B).

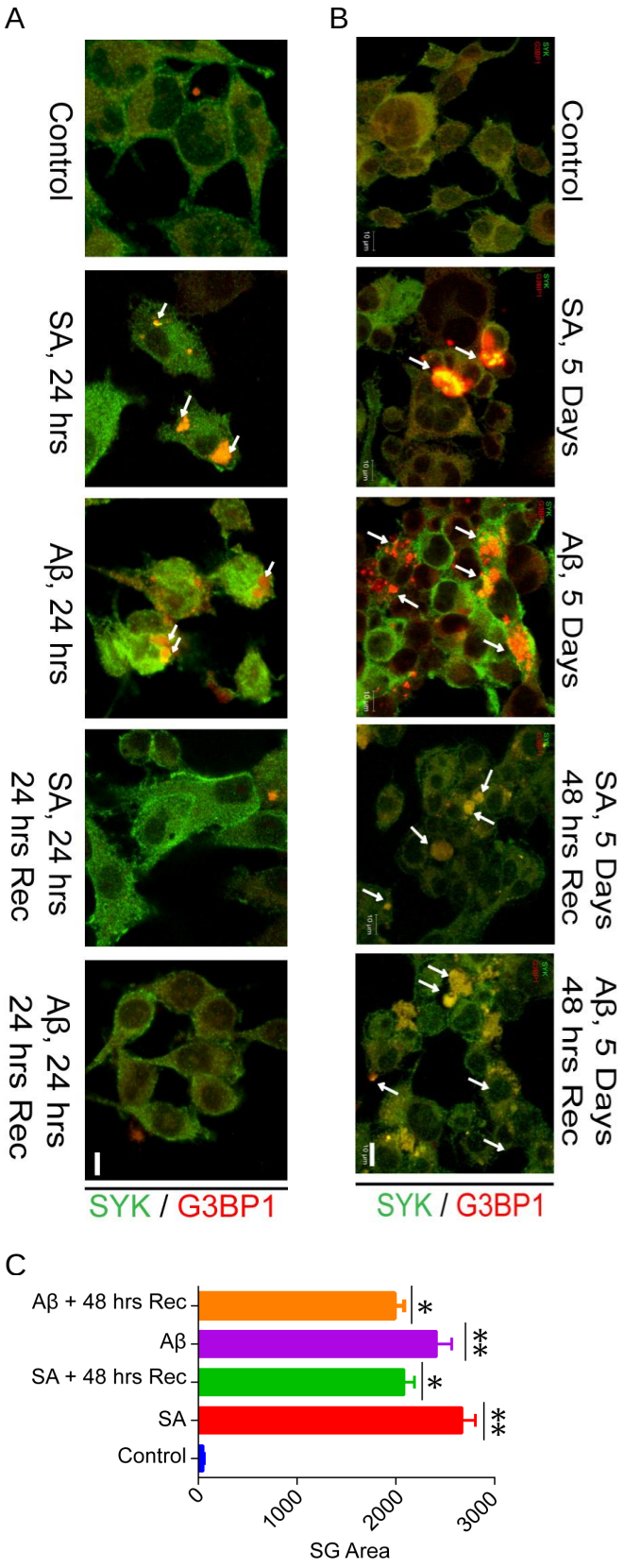

Figure 3.17 Prolonged chronic stress stimulation manifests persistent and irreversible SG in N9 cells.

Figure 3.17 (A) N9 cells were treated without (Control) or with SA or soluble Aβ for 5 days. Where indicated, cells were cultured in the absence of SA or Aβ for an additional 48 h. Cells were fixed and stained for SYK (green) and G3BP1 (red). Examples of SGs are indicated by the arrows. Bar = 10 μm. (B) N9 cells were treated for 24 h as indicated and then cultured further in the absence of any treatment for another 24 h. Cells were fixed and stained as in (A). Bar = 10 μm. (C) The area occupied by SYK and G3BP1 positive SGs from (A) was quantified by ImageJ analysis of 5 random frames from 3 independent experiments. Results represent means ± SEM. *P = 0.0001.

3.11 **Chronic stress induction leads to SYK-dependent generation of intracellular and extracellular ROS**

Activated MG can generate both reactive oxygen and nitrogen species (ROS and RNS) that can be damaging to neighboring neuronal cells (438). To determine if chronic stress led to constitutive ROS generation, I treated N9 cells with SA or Aβ for 5 days and then measured ROS production over the course of 1 h by monitoring the oxidation of cell permeant DCFDA. Cells exposed to either SA or Aβ produced substantially more ROS than control cells (Fig. 3.18A). Similar results were seen when I monitored the conversion of dihydrorhodamine 123 to rhodamine 123 as a measure of ROS production (Fig. 3.18C). Consistent with these results, cells chronically exposed to either SA or Aβ(1-42) released substantially more ROS into the media than did untreated cells (Fig. 3.18B). ROS production in cells treated with either stress stimulus was largely attenuated in the presence of the SYK inhibitor R406 (500 μM) or PRT318 (500 μM) (Fig. 3.18A, B and C).

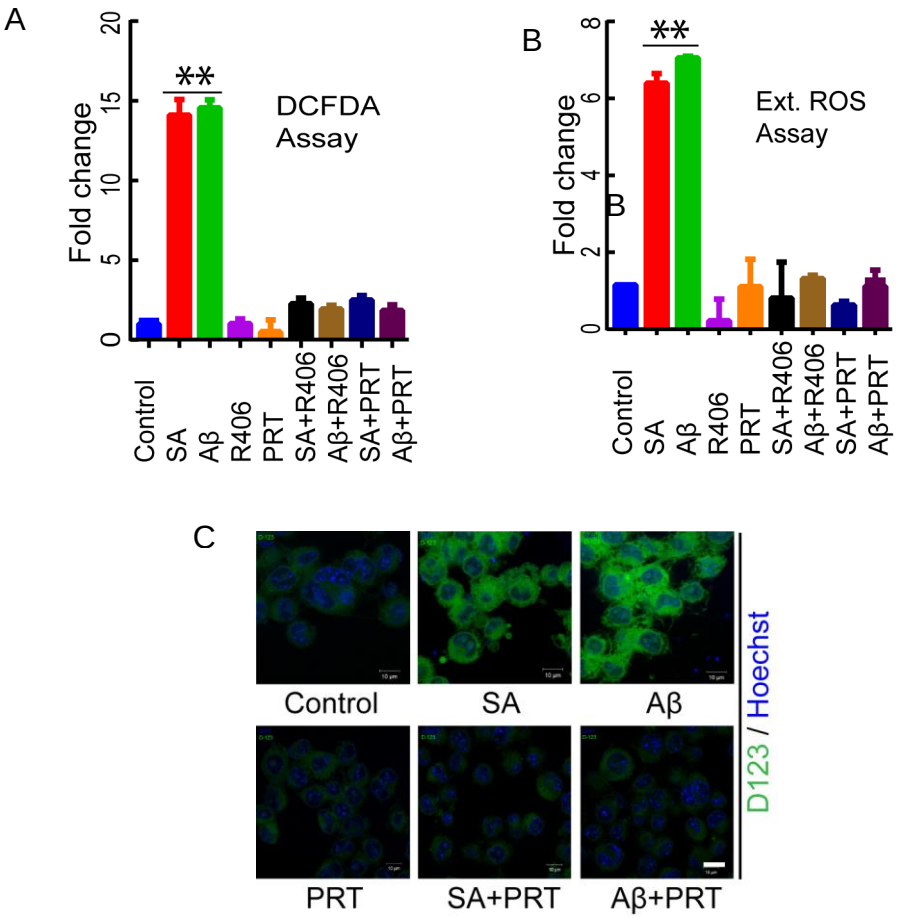

Figure 3.18 Chronic stress stimulation leads to increased ROS generation in MG cells

Figure 3.18 (A) N9 cells were treated without (Control) or with SA or Aβ for 5 days in the presence or absence of R406 or PRT318 (500 nM each). Cells were incubated with carboxy-DCFDA (10 μM) for 1 h and examined using a fluorescence plate reader. Data represent means ± SEM for 3 independent experiments. ***P* = 0.0001. (B) N9 cells were treated as in (A). Media was collected and analyzed for ROS using a chemiluminescence assay. Data represent means ± SEM for 3 independent experiments. ***P* = 0.0001. (C) N9 cells treated as in (A) and were incubated with D123 for 1 h. Green fluorescence was observed using confocal microscopy. Hoechst dye (blue) was used to stain the nucleus. Bar = 10 μm.

3.12 **Chronic stress induction leads to SYK-dependent generation of intracellular and extracellular RNS**

MG cells in AD brain acquire an activated phenotype and secrete both chemokines and NO. The induction of NO in MG is mainly mediated by overexpression of NOS2. I examined changes in the release of RNS in chronically activated MG by measuring nitrite levels in the media. Nitrite release increased three-fold from N9 cells that were chronically stimulated by SA or Aβ (Fig. 3.19B). Again, levels were reduced significantly if cells were activated in the presence of a SYK inhibitor. Since the formation of RNS in MG is mediated by NOS2 (6), I measured NOS2 levels in control, SA- and Aβ-treated N9 cells (Fig. 3.19A and B). An increase in the expression of NOS2 was observed by western blotting and immunofluorescence following both stress stimuli. The induction of NOS2 was reduced substantially in cells in which the activity of SYK was inhibited (Fig. 3.19A, B and C).

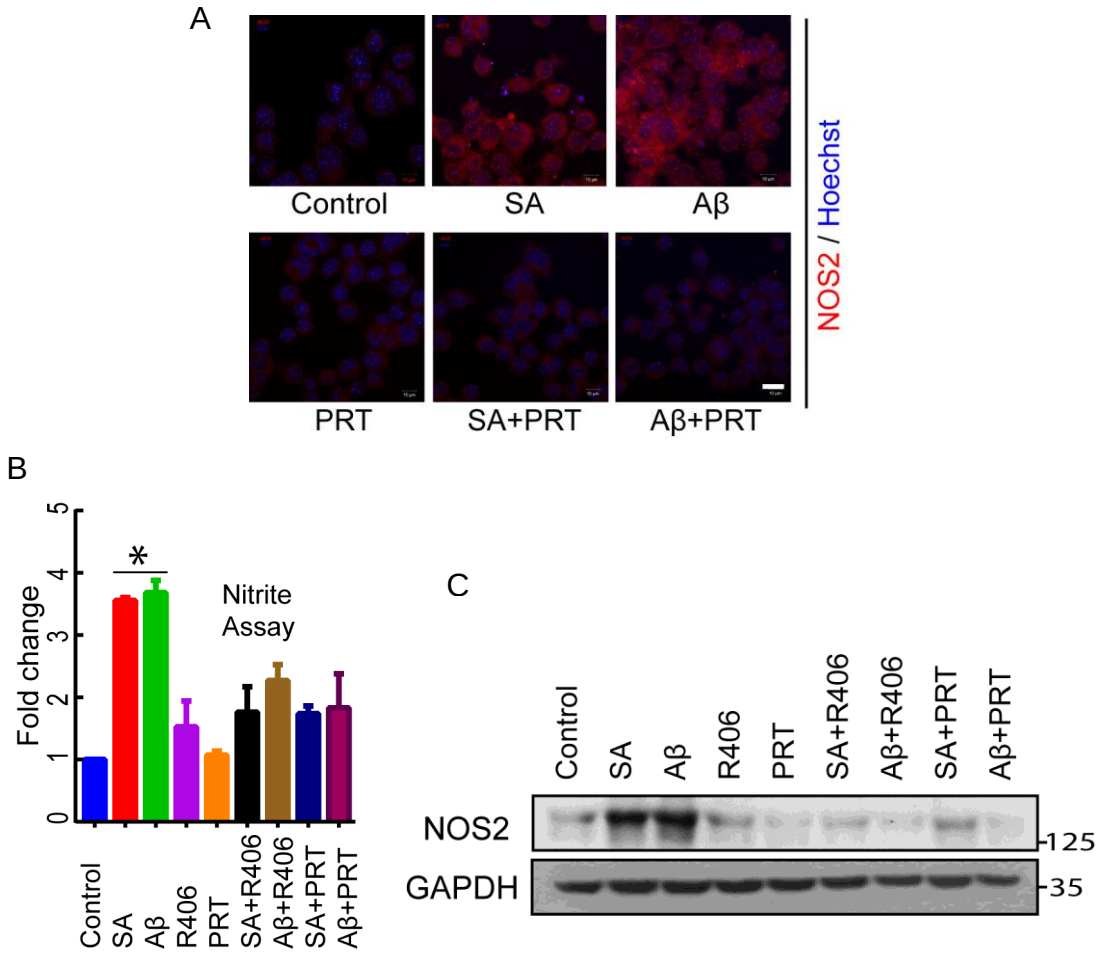

Figure 3.19. Chronic stress stimulation leads to increased RNS generation by MG cells

Figure 3.19. (A) N9 cells were treated without (Control) or with SA or Aβ for 5 days in the presence or absence of R406 or PRT318 (500 nM each). Following treatment, cells were fixed, immunostained with antibodies against NOS2 and observed using confocal microscopy. Hoechst dye (blue) was used to stain the nucleus. Scale = 10 □m. (B) N9 cells were treated as in(A). Media was collected and analyzed for nitrite levels using a fluorescent plate reader. Data represent means ± SEM for 3 independent experiments. *P = 0.001. (C) N9 cells were treated as in (A) and cells were lysed in NP-40 lysis buffer. Lysate was immunoblotted for NOS2 and GAPDH was blotted as a loading control.

3.13 **Chronic stress induced MG cells mediated neuronal cell death through ROS and RNS secretion**

Since ROS and RNS increased in chronically stressed MG, I asked if this could contribute to neuronal cell death. To test this, I co-cultured HT22 neuronal cells with N9 cells separated by a transwell insert. When chronically SA- and Aβ-treated N9 cells were cultured with healthy HT22 cells for two days, I observed a significant increase in annexin V binding to HT22 cells (Fig. 3.20A). Negligible annexin V staining was observed when HT22 cells were cultured with N9 cells that were pretreated with SA or Aβ in the presence of PRT318. To confirm an increase in ROS and RNS in the co-culture media containing activated N9 cells, I measured the levels of each generated over the two day incubation period. A substantial increase in both ROS and RNS was observed when SA- or Aβ-treated N9 cells were co-cultured with HT22 cells (Fig. 3.20B and C). The inhibition of SYK with either PRT318 or R406 reduced the external ROS and RNS levels significantly.

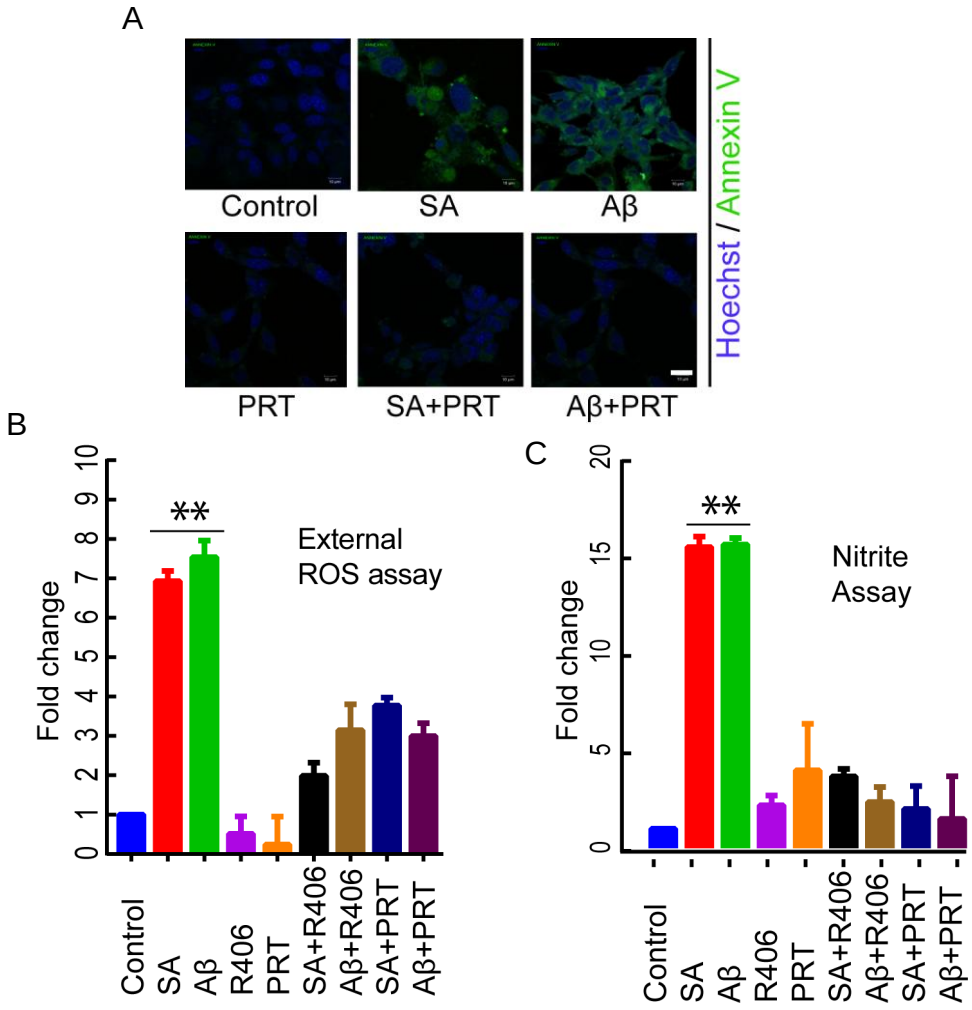

Figure 3.20. Chronically stressed MG cells induce apoptosis of HT22 neuronal cells in MG-neuron co-cultures.

Figure 3.20. (A) N9 cells were treated without (Control) or with SA or Aβ for 5 days in the presence or absence of R406 or PRT318 (500 nM each). Following treatment, N9 cells were co-cultured with HT22 for another 48 h separated by a transwell insert. HT22 cells were incubated with FITC Annexin V (green) for 30 min, fixed and imaged using confocal microscopy. Hoechst dye (blue) was used to stain the nucleus. Bar = 10 □m. (B and C) HT22 and N9 cells were co-cultured as in (A). Media was collected from the co-culture experiment and analyzed for levels of ROS (B) or RNS (C) in media using a chemiluminescence assay and fluorescence assay as describe

ed previously (Fig. 3.17B and 3.18B). Data represent means ± SEM for 3 independent experiments. ***P* = 0.0001.

3.14 **Chronic stress induction in BV-2 and N9 cells impairs phagocytosis of pH sensitive E.coli Rhodo particles**

An important function of MG that is compromised in aged and AD brains is phagocytosis. To determine if chronic stress negatively affects phagocytotic activity, I treated BV-2 or N9 cells with SA or Aβ for 5 days and then assayed the cells for the uptake of pH sensitive *E. coli* BioParticles, which fluoresce only following ingestion. Cells were either fixed and stained as shown for BV-2 cells (Fig. 3.21A) or examined by live cell imaging for N9 cells. For both cell types, chronic stress induced by either SA or Aβ strongly impaired the uptake of the pHRhodo particles. Phagocytic activity was inhibited as well when cells were treated with either R406 or PRT318 even in the absence of SA or Aβ supporting an important role for SYK in phagocytosis (Fig. 3.21A and B). Uptake of pHRhodo particles was blocked by the incubation of cells with cytochalasin D, an inhibitor of phagocytosis (Fig. 3.21C).

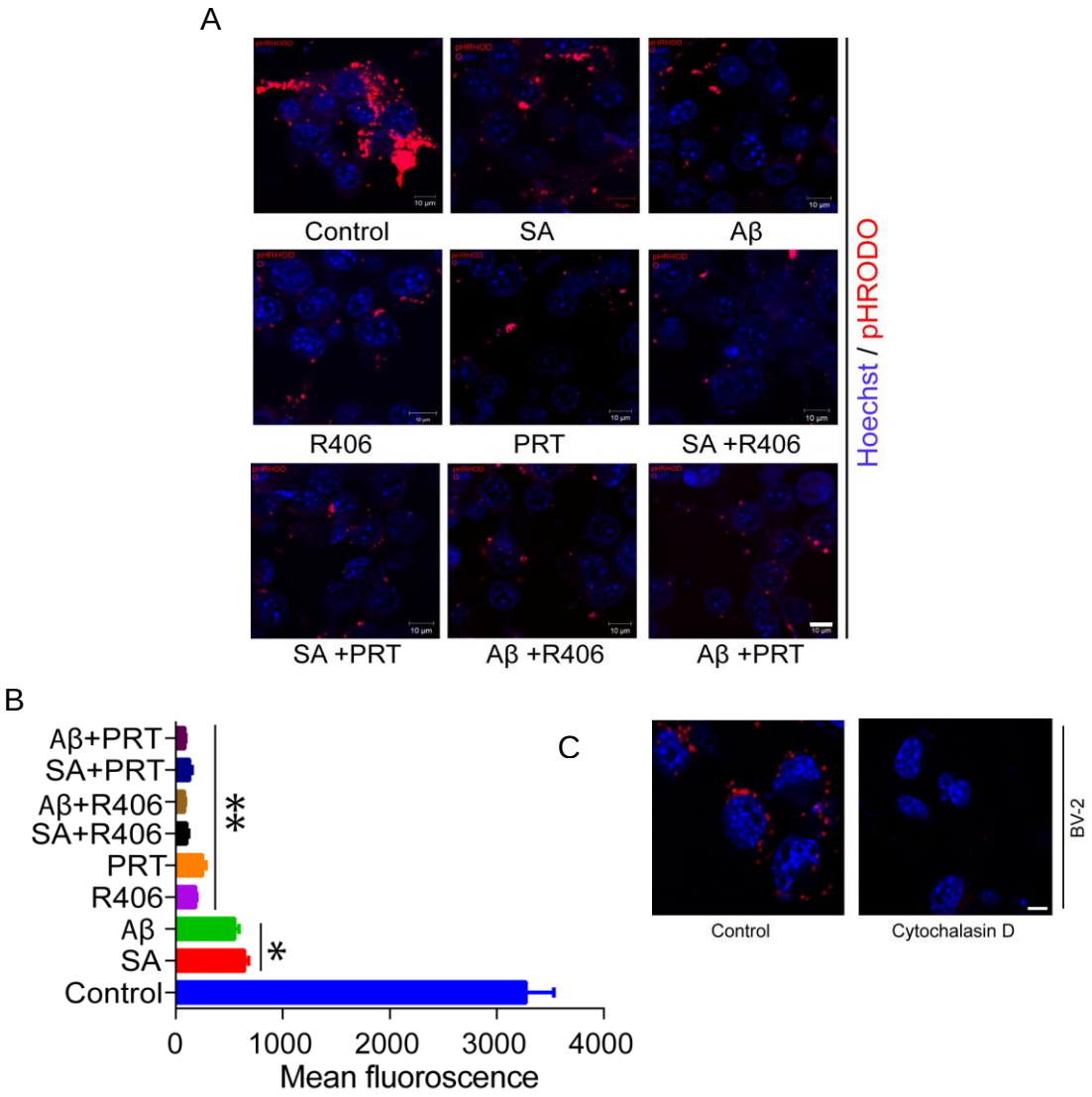

Figure 3.21 Chronic SA and Aβ stimulation and SYK inhibition impairs phagocytosis of E.coli Rhodo particles in BV-2 cells

Figure 3.21. (A) BV-2 cells were treated without (Control) or with SA or soluble Aβ for 5 days in the presence or absence of R406 or PRT381 (500 nM each) and examined for the uptake of pHRodo particles (red). Cells were fixed and examined by confocal microscopy. Hoechst dye (blue) was used to stain the nucleus. Bar = 10 µm. Similar observations were made using N9 cells examined by time lapse live cell imaging. (B) Quantification of (A) showing change in mean fluorescence measured by Image J from ten random frames in three independent experiments. *P = 0.001, **P = 0.0001. (C) N9 cells incubated for 4 h without or with cytochalasin D (10 µM) were examined for the uptake of pHRodo particles (red). Cells were fixed and examined by confocal microscopy. Hoechst dye (blue) was used to stain the nucleus. Bar = 10 µm

3.15 **Chronic stress in SG in MG prevents phagocytosis of β-amyloid fibrils**

An important function of MG is to clear β-amyloid plaques. To examine the effects of stress on this process, I treated N9 cells with SA or Aβ for 5 days and measured the uptake of fluorescein-labeled Aβ fibrils. Again, control cells readily phagocytosed Aβ fibrils whereas chronically stressed cells were unable to do so (Fig. 3.22A and B). Similar to soluble Aβ, Aβ fibrils also induced the formation of SGs containing both SYK and G3BP1 when added to either BV-2 or N9 cells (Fig. 3.22C).

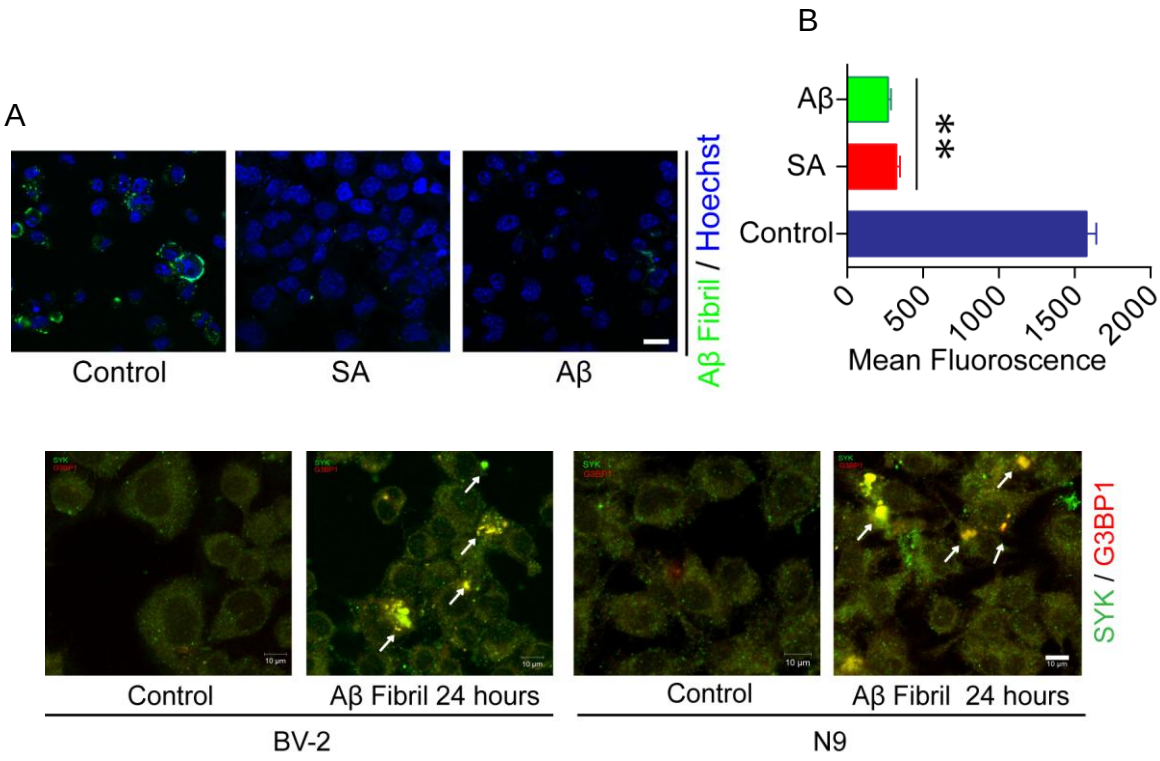

Figure 3.22 Chronic SA and Aβ stimulation impairs phagocytosis of FITC-Aβ fibrils in N9 cells

Figure 3.22. (A) N9 cells treated without (Control) or with SA or soluble Aβ for 5 days were incubated with FITC-labeled Aβ fibrils for 12 h, washed, fixed and examined by confocal microscopy. Hoechst dye (blue) was used to stain the nucleus. Scale= 10 μm. (B) Quantification of images from (A) by Image J analysis of 5 random frames from 3 independent experiments. Results represent means ± SEM. ***P* = 0.0001. (C) BV-2 or N9 cells were incubated without (Control) or with Aβ fibrils for 24 h. Cells were fixed and stained with antibodies against Syk (green) and G3BP1 (red). Only merged images are displayed. Arrows indicate the locations of SGs. Bar = 10 μm.

3.16 **SYK associated SGs are formed in brains of AD patients**

Our studies on MG indicated a connection between prolonged stress and the development of persistent SGs to which SYK was recruited. To determine if a similar mechanism was operative in AD, I examined brain samples from patients with no, mild, moderate or severe AD (Table 1). Paraffin-fixed sections of brain cortex were stained with antibodies against IBA1, SYK, G3BP1 and/or phosphotyrosine. SYK was diffusely distributed in MG in patients with no AD and in patients with mild disease, but was increasingly present in large puncta with escalating disease severity (Fig. 3.23). SYK co-localized within these puncta in diseased brain with both G3BP1 and phosphotyrosine indicating that the kinase was localized to SGs and was active at these sites (Fig. 3.24 and 3.25).

Table 1. Characteristics of Alzheimer's disease patients used to measure SYK, G3BP1 and phosphotyrosine (pTYR) co-localized in SGs.

Disease State	No. of patients	Avg. Age	Avg. MMSE score	Total plaques	Total tangles	Area covered by SG Puncta (SYK & G3BP1) Fluorescence	Area covered by SG Puncta (SYK & pTYR) Fluorescence
Normal	6	77	30	3	2	148	234
Mild	6	91	26	1.5	4	268	435
Moderate	6	91	22	7	5	675	746
Severe	6	82	10	14	14	1357*	1467*

The mean area occupied by SGs positive for SYK and G3BP1 or SYK and pTYR was quantified by Image J analysis of 5 random frames from 6 different patient slides of each disease state. *P = 0.001.

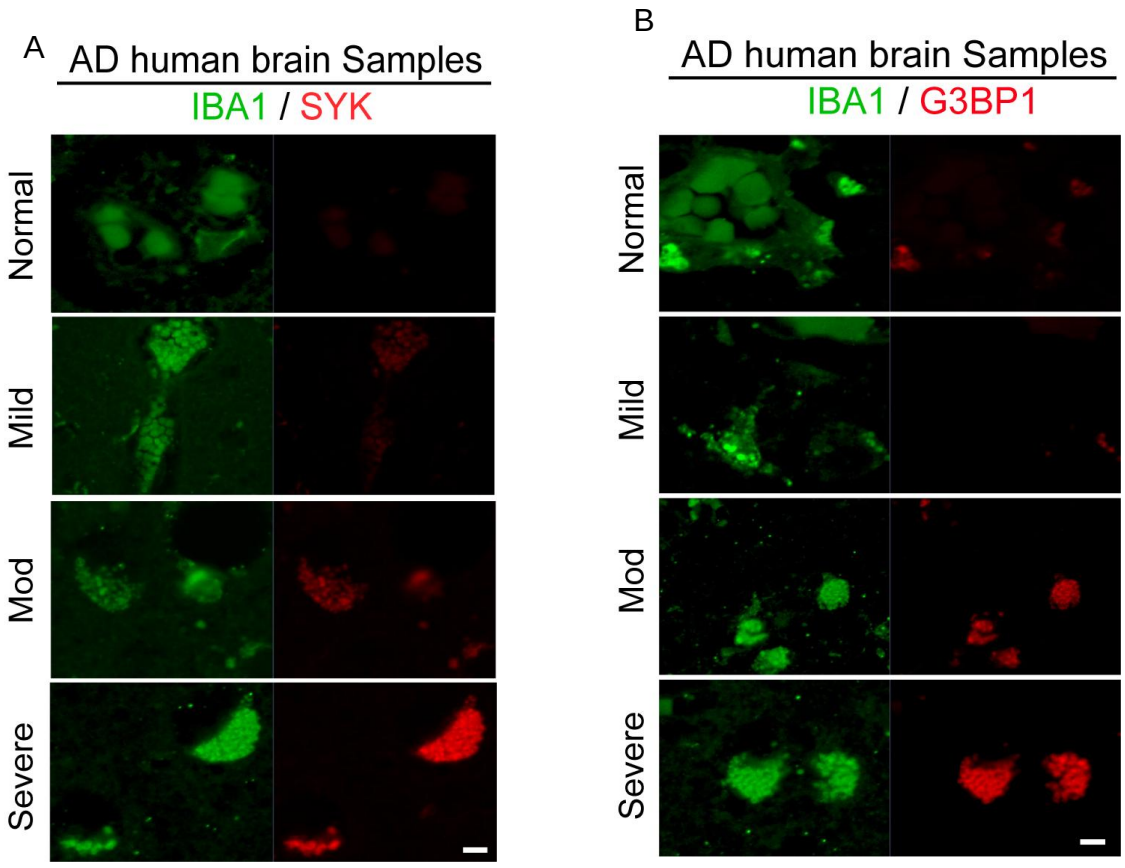

Figure 3.23. SYK and G3BP1 positive granules were observed in human AD brain samples

Figure 3.23 (A and B) Paraffin embedded slides of human brain cortex from patients with no (Normal), mild, moderate (Mod) and severe AD were immunostained for IBA1 (microglia marker), SYK and G3BP1 independently as indicated. Bar = 5 μm.

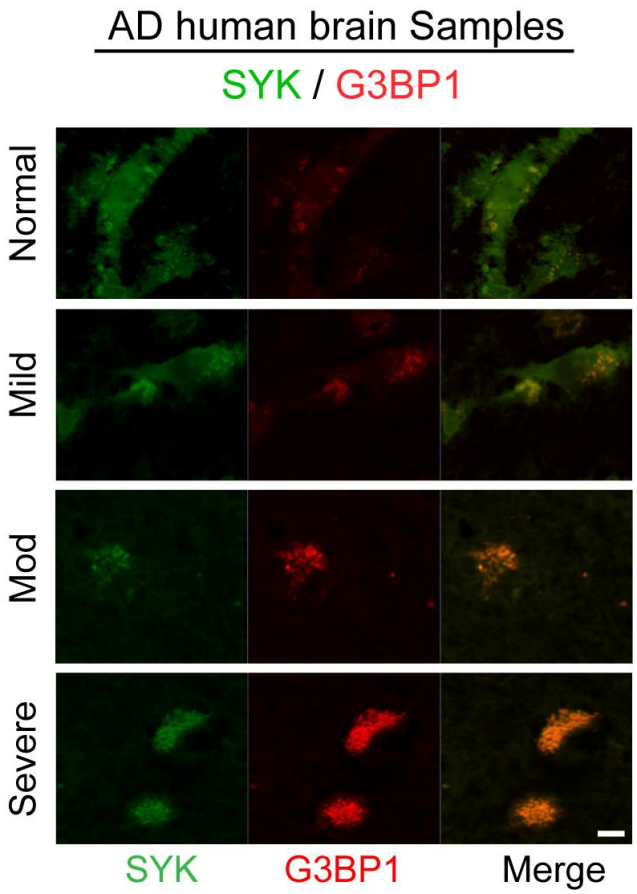

Figure 3.24 SYK and G3BP1 positive granules co-localized in human AD brain samples

Figure 3.24 Paraffin embedded slides of human brain cortex from patients with no (Normal), mild, moderate (Mod) and severe AD were immunostained for SYK and G3BP1 as indicated. Bar = 5 μm.

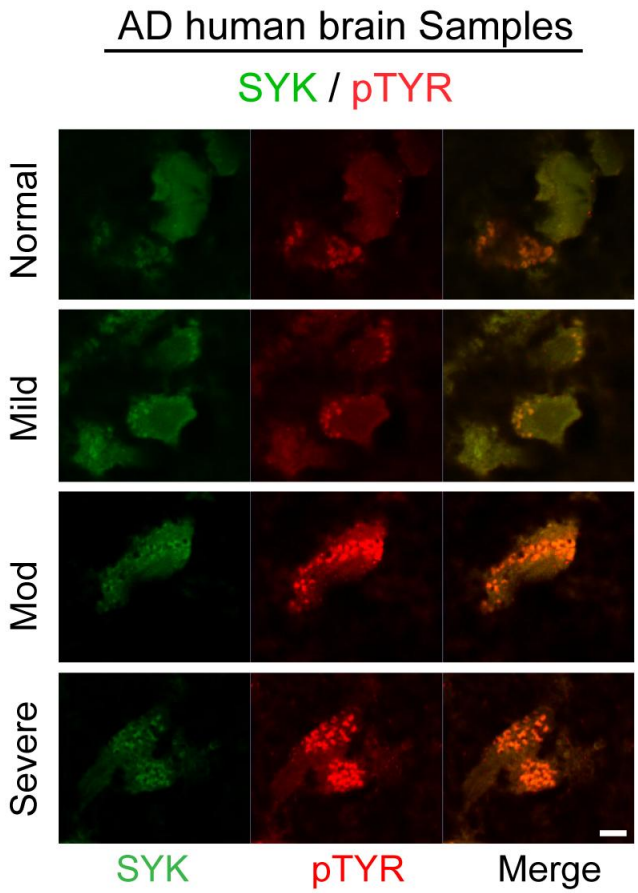

Figure 3.25 SYK positive granules co-localized with phosphotyrosine in human AD brain samples

Figure 3.25 Paraffin embedded slides of human brain cortex from patients with no (Normal), mild, moderate (Mod) and severe AD were immunostained for SYK and phosphotyrosine as indicated. Bar = 5 μm.

3.17 **IgG treatment can repair impaired phagocytosis of MG after chronic stress induction.**

Since phagocytosis of MG was impaired under SA or Aβ stimulation, I wanted to devise a way for cells to recover from phagocytosis impairment. I asked if manipulations of SYK activity or localization might restore the ability of chronically stressed MG to phagocytose foreign particles. Since integrin ligation leads to the activation of SYK in macrophages (439), I first treated chronically stressed N9 cells with rabbit IgG directed against β1 integrin. Treatment of stressed N9 cells for 4 h led to a marked recovery of phagocytic activity (Fig. 3.25). As a control, I treated stressed N9 cells with nonspecific rabbit IgG isotype control antibodies. Interestingly, phagocytic activity again was restored. To explore this further, I treated stressed cells with an affinity purified rabbit IgG directed against a peptide from the cytoplasmic C-terminus of the inhibitory receptor CD32, an antibody that should neither react with intact MG nor activate SYK. Again, incubation with this antibody led to a recovery of phagocytic activity in chronically stressed cells (Fig. 3.25). Thus, phagocytic activity in stressed MG could be restored by treatment with IgG independent of the specificity of the antibody.

To explore the mechanism further, I examined the effects of IgG on the localization of SYK. Treatment of N9 cells with either non-specific rabbit IgG or the anti-CD32 antibody led to a relocalization of SYK from the cytoplasm to the plasma membrane, the site where phagocytosis takes place. Even in chronically stressed cells, a substantial amount of SYK was translocated from SGs to the membrane (Fig. 3.26). Western blotting

of detergent-soluble and -insoluble fractions of N9 cells confirmed the partial re-location of SYK from the insoluble fraction in which SGs are located to the detergent-soluble fraction following incubation with rabbit IgG or anti-CD32 (Fig. 3.27A and B).

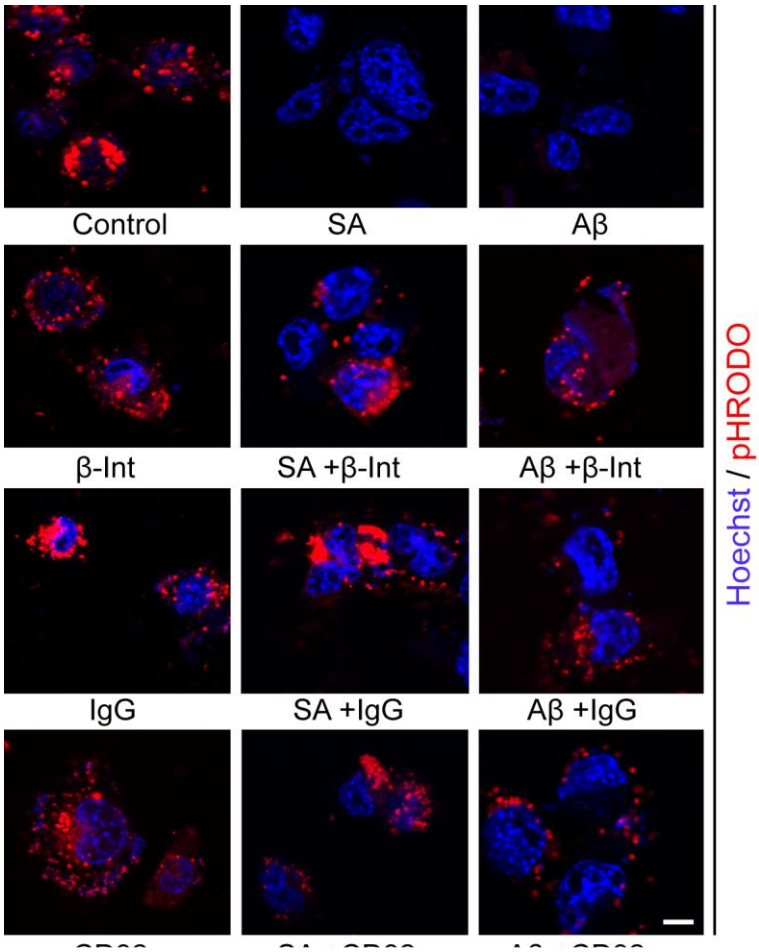

Figure 3.26 IgG treatment of chronically stressed N9 cells restores phagocytic activity.

Figure 3.26. N9 cells were treated without (Control) or with SA or soluble Aβ for 120 h. At 116 h, to one set of control, SA- and Aβ-treated cells were treated with anti-β1-integrin (β-Int), mixed rabbit IgG (IgG) or anti-CD32 for the final 4 h. Cells were then incubated with pHRhodo particles (red) to measure phagocytosis. Cells were fixed and stained with Hoechst dye (blue) to visualize the nucleus. Bar = 10 μm.

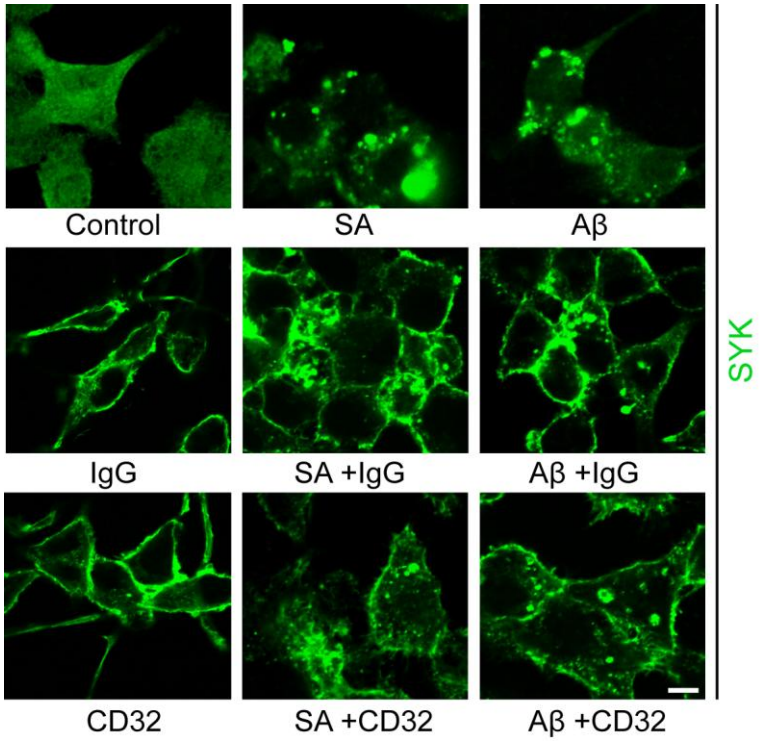

Figure 3.27. IgG treatment of chronically stressed N9 cells relocalizes SYK to the plasma membrane

Figure 3.27. N9 cells were treated without (Control) or with SA or soluble Aβ for 120 h in the presence or absence of R406 or PRT318 (500 nM each). At 116 h, to one set of control, SA- and Aβ-treated cells were treated with mixed rabbit IgG (IgG) or anti-CD32 for the final 4 h. Cells were then fixed and stained with antibodies against endogenous SYK. Bar = 10 μm.

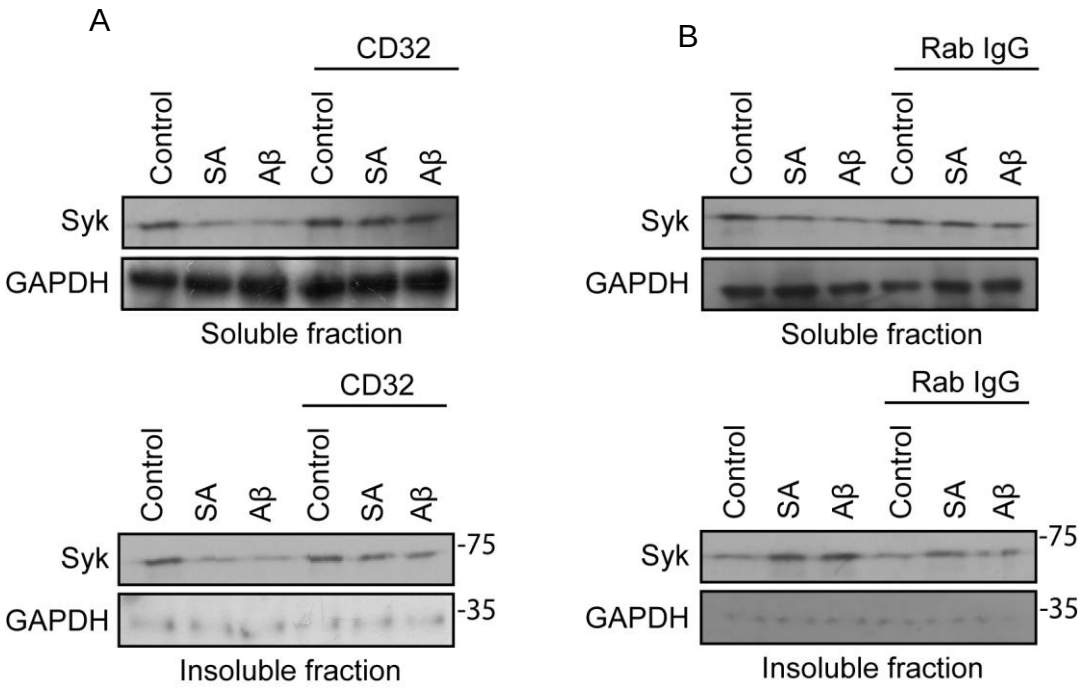

Figure 3.28 IgG treatment of chronically stressed N9 cells partially relocalizes SYK from the SG insoluble fraction to the soluble fraction.

Figure 3.27 (A and B) N9 cells were treated without (Control) or with SA or soluble Aβ for 120 h. At 116 h, one set of control, SA- and Aβ-treated cells were treated with anti-CD32 (A) or rabbit IgG (Rab IgG) (.B) for the final 4 h. Cells were separated into detergent soluble and insoluble fractions, which were analyzed by western blotting for SYK and GAPDH.

CHAPTER FOUR: DISCUSSION

Paradoxically, MG from aged and AD brain exhibit a phenotype that combines a perpetually activated state with functionally impaired phagocytic activity (3, 6). To explain this dichotomy, we propose a model in which the altered activity and localization of SYK contributes to both phenotypes. In this model, chronic stress leads to the formation of SGs to which SYK is recruited resulting both in a persistent pro-inflammatory phenotype and impaired phagocytosis.

SG formation is thought to be, at least initially, a defense against stress, providing a mechanism for the selective sequestration and protection from degradation of a subset of mRNAs (27). However, it is clear that persistent formation of RNP particles is deleterious to cells as noted for several neurodegenerative disorders where aggregates appear in neuronal cells (31, 40). Our data indicate that SGs form, as well, in MG. In fact, MG appears particularly susceptible to the stress-induced formation of SGs as their induction requires exposure only to low levels of SA. The recruitment of SYK to SGs in non-inflammatory cells protects them from stress-induced damage by stimulating SG clearance through autophagy, an event that requires both SYK activity and its association with SGs (Krisenko et al., manuscript submitted for publication). This process may be operative as well in MG as SGs can be cleared effectively from MG exposed to stress for short periods of time. However, prolonged stress leads to the formation of large SGs that

associate with SYK and are resistant to clearance as they persist following removal of the stimulus. SYK in SGs is active as reflected by the presence of phosphotyrosine-containing proteins, which is a function of SYK's catalytic activity (Krisenko et al., manuscript submitted for publication). Large SGs also are characteristic of MG in the brains of patients with advanced AD, and these also contain abundant SYK and phosphotyrosine. Thus, the appearance of SYK- and phosphotyrosine-containing puncta in brain samples may serve as a useful marker of dysfunctional MG in neurodegenerative disease.

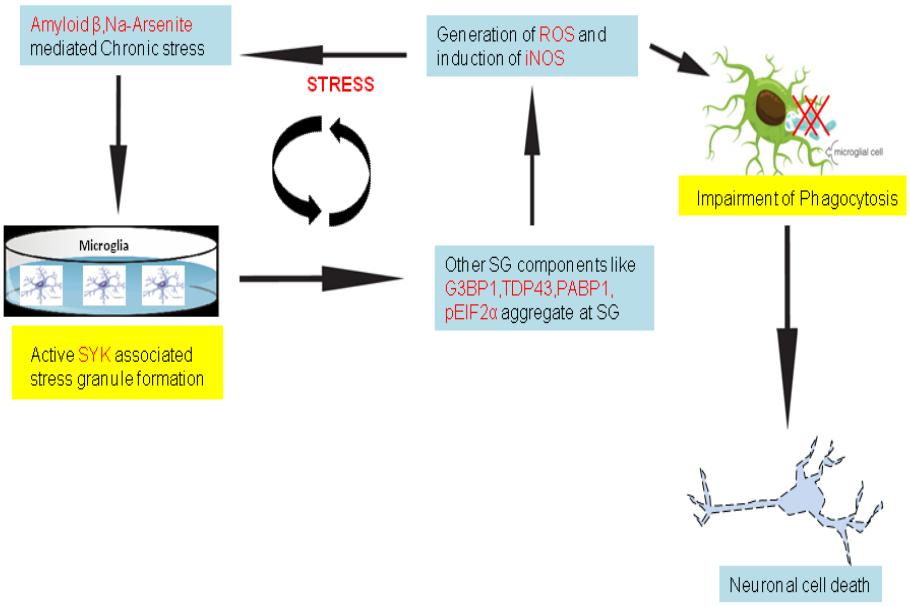

Figure 4.1 Model of SYK associated stress granules formation in microglial cell

SA induces SG formation in part through the generation of ROS[41]. Oxidative stress also is thought to precede the pathogenesis of AD since enhanced neuroinflammation resulting from traumatic brain injury, infection, or aging predispose individuals to AD while the long-term use of NSAIDS can be protective (440). We find that when stress stimulates the formation of SGs in MG this hampers their ability to phagocytose Aβ(1-42) fibrils. Thus, we predict that enhanced accumulation of Aβ plaques in the brain would be an expected result of a prolonged exposure to oxidative stress. We find further that the exposure of MG to Aβ or Aβ fibrils alone also induces the appearance of abundant SGs. This is consistent with the known ability of Aβ to trigger MG cell activation and promote the generation of ROS and RNS (9, 376). Thus, a defect in the removal of Aβ fibrils by MG compromised by oxidative stress could cause a build-up of Aβ plaques leading to additional Aβ-induced stress and the enhanced formation of persistent SGs. MG from aged mice are more sensitive to the stress-induced formation of SGs suggesting one mechanism by which age might lead to a greater susceptibility to AD.

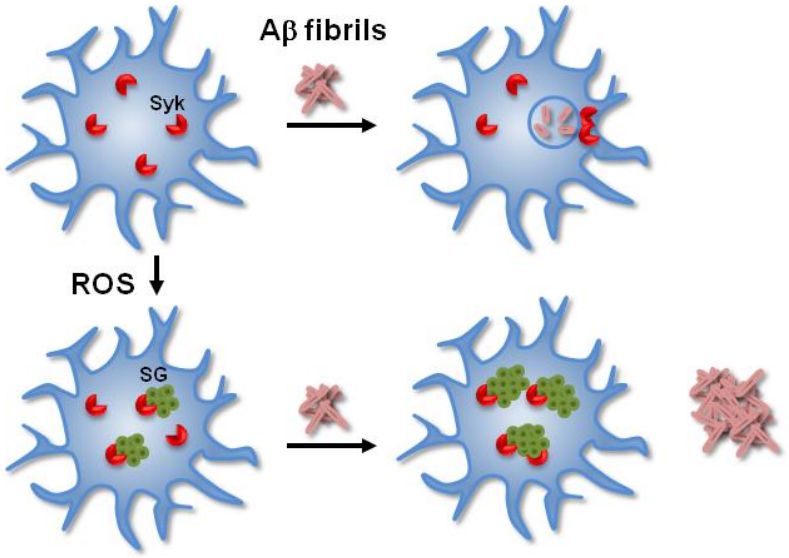

Figure 4.2 Graph art depicting stress granule formation in microglial cell upon Aβ stimulation

The formation of SGs in MG in response to low levels of either SA or Aβ requires the expression of SYK as it is blocked by SYK inhibitors or by a knockdown or reduction of SYK expression. In inflammatory cells like MG, SYK plays a well known role in the receptor-mediated production of ROS and RNS (271). Aβ binds to MG via an assortment of cell surface receptors (6) many of which, including CD14, TLR4, CD36, and SIRPβ1, are coupled to SYK either directly or through receptor-associated proteins that contain ITAMs (254, 367, 383, 441, 442). SYK also is activated in cells under conditions of increased oxidative stress (443). The resulting SYK-dependent pro-inflammatory response may be further exacerbated by the retention within SGs of active SYK, which may continue to stimulate down-stream signaling pathways that generate ROS or RNS. The production of ROS and RNS can be damaging to neighboring cells (6) and we find, in fact, that chronically activated MG produce mediators that are toxic to HT22 neuronal cells. Consistent with a role for SYK in these processes, the generation of chronically activated MG that produce ROS and RNS and the ability of these cells to kill neuronal cells are all blocked by SYK inhibitors.

The ability of MG to clear Aβ plaques through phagocytosis is important for the control of AD (5, 439). We find that chronic stress induced by prolonged exposure of MG to either SA or Aβ inhibits the ability of these cells to phagocytose either bacteria or Aβ fibrils. Interestingly, in a mouse model of AD, the phagocytic activity of MG also is selectively compromised in brain regions containing Aβ plaques (4). We find that chronic stimulation of MG with either SA or Aβ results in the formation of very large SGs to which SYK is recruited and propose that it is the sequestration of the kinase away from

phagocytic receptors that underlies the impaired phagocytic activity of MG encountering Aβ plaques.

A role for SYK in both phagocytosis and the activation of MG complicates the development of strategies for the use of specific SYK inhibitors for the treatment or prevention of AD. However, the finding that active SYK is sequestered in SGs suggests that strategies to relocate the enzyme might be effective in restoring some function to damaged MG. We find that the treatment of chronically stressed MG with rabbit IgG leads to a dramatic restoration of phagocytic activity. The recovery of activity is not a function of the reactivity of the IgG to any specific antigen as even an affinity-purified antibody prepared against a peptide found on the cytoplasmic domain of an ITIM-containing receptor is able to induce this effect. Treatment of MG with IgG leads to a relocalization of SYK from the cytoplasm to the plasma membrane in unstressed cells and from SGs to the membrane in chronically stressed cells. This effect may help explain the therapeutic benefits of intravenous immunoglobulins (IVIg), which have shown efficacy in slowing the progression of AD in some human clinical trials (444-446). The benefits of IVIg are generally attributed to the presence of anti-Aβ antibodies in the pooled IgG preparations (444). However, it is interesting to note that the administration of pooled mouse IgG having no detectable anti-Aβ activity to the brains of APP/PS1 mice leads to a similar reduction in Aβ deposits as does IVIg (447). Thus, even immunoglobulins that fail to recognize Aβ have the potential to reverse defects in the function of stressed MG. This observation suggests that the development of IgG-related therapeutics optimized for the recovery of phagocytic activity of MG would be an attractive strategy for augmenting current strategies for the treatment of AD.

CHAPTER FIVE: FUTURE DIRECTIONS

Stress granule formation in neurodegenrative brain has been observed in several diseases including ALS, AD, SMA and FXMR(29). Autopsy studies on the brains of these patients have indicated an increased aggregation of SG-associated proteins with increasing severity of the disease. Our studies showed for the first time the presence of SGs in MG. MG over-activation is the leading cause of neuroinflammation in neurodegenerative diseases. Our data show that SYK promotes SG formation in MG under SA or Aβ mediated stress. It would be very interesting to further dissect the other stressors that influence the formation of SGs in MG. We know, for example, that several genetic mutations in genes like *tau*, *PINK1*, *PARKIN*, *APP*, *SNCA*, *CDK5*, *MST1* and *prion* directly manifests disease symptoms (448, 449). To better understand the role of SGs in these diseases, it would be interesting to examine these mutant proteins to determine if they directly or indirectly drive SG formation or inhibit SG clearance in the brain during disease progression.

In this study, we discovered that chronic stress at a very low dose can induce SGs that are persistent and cannot be removed upon removal of stress. We also observed that SYK localizes in SGs and influences their formation in these cells. Additionally, SYK regulates ROS and RNS production of MG cells. Many pathways dissected in AD have

indicated that excessive production of ROS and RNS hastens neuronal cell death through chronic neuroinflammation. Our study also showed that SYK associates with other misfolded proteins like TDP-43 upon stress stimulation. Syk was also observed to be active in these SGs. SYK is a tyrosine kinase that phosphorylates several downstream proteins to trigger activation of other pathways such as Ras/ERK and activate transcription factors such as NF-κB (138, 224, 450). These downstream proteins directly or indirectly modulate release of pro-inflammatory factors, cytokines or reactive species in various cells including immune cells. A proteomic screen of MG under acute or chronic stress will help to identify the proteins that associate with SYK in SGs under chronic stress. Furthermore, we can also identify the major pathways that are activated that result in SG formation. It is well known that SYK associates with ITAM bearing receptor proteins in MG. A detailed tyrosine phosphoproteomic screen could identify the receptors that activate SYK under SG formation conditions. Several MG receptors like FcγR, integrins, CD33 and CD-3 are involved in engulfing Aβ plagues and tau tangles in AD (341, 451). The screening could also reveal the receptors that lose their ability to engulf foreign particles like misfolded proteins under chronic stress.

Autophagy is a mechanism by which cells get rid of intracellular protein aggregates and damaged organelles. The intracellular proteins are engulfed by double walled vacuoles and delivered to lysosomes for degradation. In neurodegenrative diseases, studies have shown that autophagy mediates removal of impaired organelles and toxic protein aggregates like HTT, tau and beta amyloid. Impairment of autophagy is also observed in neurodegenrative diseases like AD, PD and HD (32, 410). Additionally, several studies suggest that promoting autophagy alleviates disease symptoms in AD and

HD. On the other hand, it has been reported in a few studies that autophagy could be one of the mechanisms by which SGs are cleared from the cell. Based on such evidence, it could be proposed that impairment of autophagy leads to an increase in the number of pathological SGs in neurodegenrative brain. Due to reduced autophagy, a greater number of toxic protein aggregates are sequestered to the SGs and a chain of downstream unfavorable reactions occur. Understanding the connection between autophagy and SGs in neurodegenrative brain, specifically MG, could provide us with better therapeutic approaches to retarding disease progression. It would be also interesting to examine if promoting autophagy would increase clearance of pathological SGs resulting in alleviated disease symptoms.

One of the vital functions of MG is phagocytosis of protein aggregates, debris and dead cells. MG efficiently perform phagocytosis of amyloid plagues and tau fibrils in early AD brain, but fail to do so with disease progression. Additionally MG adopt an activated state, releasing cytokines, chemokines and reactive species contributing to inflammation (124, 430). In our study, we have shown that inhibition of SYK in Aβ stressed MG can prevent the generation of such reactive species. Sadly, SYK inhibition also impairs phagocytosis. But another finding in our study also showed that IgG treatment can re-localize SYK partially to the plasma membrane from SGs. This re-localization reconstituted the phagocytosis machinery in MG and the number of SGs also was reduced. Further studies need to be done to dissect which receptors IgG binds to on the membrane to provoke such a relocalization of SYK. It would be interesting to determine if the binding of IgG Fc regions to the receptors is enough to relocalize SYK and if specific Fcγ receptors are required for this process. A knowledge of IgG binding

and SYK relocalization could prove to be a promising therapeutic approach for repairing MG in AD.

Since this study led to some interesting findings about SYK's role in MG activation in AD; it would be logical to expand this study to neurodegenrative animal models. APP/PS1 is a very suitable mouse transgenic model to study Aβ mediated neurodegeneration in AD (131, 222). Our study suggests that SYK inhibition and IVIg can be a promising therapeutic approach to repair impaired MG in AD. An animal model study involving use of SYK inhibitors and IVIg independently or in combination in APP/PS1 transgenic mice would be interesting. Examination of neuroinflammation and MG activation in those animal models will tell us more about microglial SYK's role in AD. Additionally, behavioral studies would also help us determine the importance of this study in the development of therapeutics.

Primary MG when stressed with SA or Aβ developed large SYK and G3BP1 positive SGs. MG from 20 month old mice showed large SGs compared to 1 month old mice. Further examination in SYK haplosufficient mice showed reduced SGs in stressed MG confirming SYK's role in SG formation. Since the SYK knockout mouse is biologically lethal, we are trying to develop a transgenic mouse that expresses an analog sensitive form of SYK (296). Treatment with an orthogonal inhibitor like NM-PP1 can inhibit SYK's activity selectively in these mice at any point in their life cycle (452). It would be interesting to examine the SG dynamics in MG of these mice upon Aβ stress induction. Subsequent investigation of MG in this genetically modified SYK mouse could give us insights into receptors that are impaired upon SYK knockdown in the long run. We could also detect various behavioral or pathological implications of SYK

knockdown in an adult mouse. The next step would be to develop a transgenic mouse by genetically crossing analog sensitive form of SYK knockout mouse and APP/PS1 mice. Such transgenic mice can further help us to dissect the exact role of SYK in overall progression of AD. By turning off SYK at any specific time point in the life cycle of such transgenic mice, we can identify the role of microglial SYK in early and late AD. We can also determine the various SYK associated ITAM receptors that are activated and impaired at different stages of AD.

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VITA

VITA

Soumitra Ghosh was born on April 20, 1987 in Mumbai, India. He moved to Kolkata soon after and finished his schooling at Kendriya Vidyalaya Cossipore. Right from childhood he dreamt about becoming a scientist. Among all the science subjects he learned as a kid, biology appealed him the most. To fulfill his dreams he decided to major in Genetic Engineering as an undergraduate. Four years later in 2009, Soumitra completed his Bachelor of Technology degree in Genetic Engineering from SRM University, Chennai, India. During his undergraduate to obtain research experience, he spent all his summer vacation as an intern in various Universities and national labs. In his junior year, he managed to get a summer fellowship from Department of Biotechnology, India to pursue neuroscience research at ACBR, Delhi. He soon realized that neuroscience was his area of interest and decided to obtain a PhD. in neuroscience. In August 2009 right after graduation, he joined Purdue University as a graduate student to pursue his neuroscience research. Initially he worked in Department of Chemistry and later moved to Geahlen lab. Under excellent guidance and support of Dr. Robert Geahlen, he studied the role of Syk in microglial cell and stress granules. In the fall of 2015 he completed his PhD in Medicinal Chemistry and Molecular Pharmacology with a concentration in integrative neuroscience. He is going to continue as a post doctoral researcher in neuroimmunology and chase his dreams of becoming an independent scientist.

PUBLICATIONS

PUBLICATIONS

Ghosh S and Geahlen LR. Stress Granules modulate Syk to cause Microglial Dysfunction in Alzheimer's disease. 2015 (EBioMedicine , In press, September 2015)

Krisenko MO, Higgins RL, Ghosh **S** Zhou Q, Trybula JS, Wang WH, and Geahlen RL. Syk is Recruited to Stress Granules and Promotes their Clearance through Autophagy. (The Journal of Biological Chemistry, In press, October 2015)

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